The Amelioration of Transplantation associated Ischaemia Reperfusion Injury by the novel Heme Oxygenase-1 inducer Heme Arginate

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For my parents, Frances and Robert

Abstract

A kidney transplant is the optimum treatment for patients with renal failure, both prolonging life and improving its quality (Parfrey, 2000. Wolfe, 1999). Transplanting an organ subjects it to Ischaemia reperfusion injury (IRI) as the vascular supply is temporarily disrupted and then reinstated. IRI is a risk factor for delayed graft function (DGF) (Bronzatto, 2009), which prejudices both short and long-term graft survival (Ojo, 1997). The severity of IRI has also been linked with early changes within the renal microvasculature that correlate with subsequent impaired organ function and DGF following transplantation (Schmitz, 2008). At the current time there are no specific treatments for IRI. Heme oxygenase-1 (HO-1) is a 32kDa enzyme that catalyses the breakdown of Heme molecules to Biliverdin, Carbon monoxide and free Iron. Studies suggest that induction of HO-1 prior to surgery may be beneficial by reducing the severity of injury in animal models of IRI and transplantation (Amersi, 1999. Tullius 2002). The majority of such studies utilise heavy metal protoporphyrins as HO-1 inducing agents. These substances are highly toxic, preventing their use in clinical practice. Heme arginate (HA) is a clinically licensed drug used in humans for the treatment of porphyria. It is well tolerated, with a minimal side effect profile (Mustajoki, 2003). It has also been shown that HA can induce HO-1 in healthy human volunteers (Doberer, 2010). The principal aims of this thesis are to determine whether the administration of HA can confer protection within *in vitro* and *in vivo* models of IRI and renal isograft transplantation. I assess the structural integrity of the microvasculature and evaluate changes in the inflammatory cell populations following transplantation and IRI to determine whether these correlate with the degree of renal tubular injury observed.

Initial *In vitro* experiments demonstrate the potential for HA to induce HO-1 in a murine cardiac endothelial cell line (MCEC-1). I subsequently develop and characterise an *in vitro* model that simulates the changes in gaseous tensions encountered during IRI *in vivo,* and show that MCEC-1 cells that are pre-treated with HA are significantly protected against exposure to these adverse conditions ($n=3$, $p<0.05$).

In subsequent *in vivo* experiments, HA administration was shown to up-regulate functional HO-1 within murine renal tissue. Mice that were pre-treated with HA 24 hours prior to surgery showed significant preservation of renal tubules and renal function (creatinine) in a murine model of renal IRI ($n=8$, $p<0.05$). In a series of murine renal isograft transplants, HA pre-treatment of organ donors resulted in significant protection of renal tubules against IRI ($n=10$, $p<0.05$). An apparent, but statistically insignificant, trend toward protection of renal tubules was also observed in organ recipients that were pre-treated with HA ($n=10$, $p=0.21$). Further experiments are necessary to clarify the underlying mechanisms responsible for the apparent protective effects of HA against tubular injury *in vivo*.

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Declaration

I, Matthew Frederick Beesley, declare that this thesis has been composed by myself and that the work contained herein is my own. The work was performed with the technical help of the people I have acknowledged in the appropriate section and has not been submitted for any other degree or professional qualification.

Matthew Beesley

Abbreviations

%	:	Percentage
°C	:	Degrees Celsius
ACE	:	Angiotensin Converting Enzyme
AKI	:	Acute Kidney Injury
ATP	:	Adenosine Triphosphate
BMDM	:	Bone Marrow Derived Macrophages
BSA	:	Bovine Serum Albumin
BUN	:	Blood Urea Nitrogen
cAMP	:	Cyclic Adenosine Monophosphate
CD31	:	Cluster of Differentiation 31
CEC	:	Circulating Endothelial cells
CO	:	Carbon Monoxide
CO_2	:	Carbon dioxide
CORM	:	Carbon Monoxide Releasing Factor
DAB	:	Diaminobenzidine
DAPI	:	4',6-diamidino-2-phenylindole
DGF	:	Delayed Graft Function
DMEM	:	Dulbeccos Modified Eagles Medium
DMSO	:	DiMethyl Sulphoxide
DNA	:	Deoxyribonucleic acid
DT	:	Diptheria toxin
ECGF	:	Endothelial Cell Growth Factor
ECL	:	Enhanced Chemiluminescence
EDTA	:	Ethylenediaminetetraacetic acid

EaHy926 : Human Umbilical Vein cell line

ECV 304 : A human endothelial cell line

eNOS : Endothelial nitric oxide synthase

EPO : Erythropoietin

ESRF : End Stage Renal Failure

ET-1 : Endothelin-1

EVNP : Ex Vivo Normothermic Perfusion

FCS : Foetal Calf Serum

fDGF : Functional delayed graft function

GFR : Glomerular Filtration Rate

H_20_2 : Hydrogen Peroxide

H&E : Haematoxylin and Eosin

HA : Heme arginate

HCL : Hydrochloride

HCR : Hypoxia and Hypercarbia (0.5% O2 and 11.5% CO2)

HIF-1α : Hypoxia Inducible Factor One Alpha

HMEC-1 : Human Dermal Microvascular Endothelial Cell Line

HO-1 : Heme oxygenase-1

HO-2 : Heme oxygenase-2

HO-3 : Heme oxygenase-3

HPF : High-powered Fields

HRP : Horseradish Peroxidase

HTK : Histidine-Tryptophan-Ketoglutarate solution

ICAM-1 : Intercellular adhesion molecule 1

IFNγ : Interferon Gamma

Ig	: Immunoglobulin
IgG	: Immunoglobulin G
IL	: Interleukin
IL-1β	: Interleukin 1 Beta
IL-4	: Interleukin 4
IL-10	: Interleukin 10
i.p.	: Intraperitoneally
iNOS	: Inducible Nitric Oxide Synthase
IRI	: Ischaemia reperfusion injury
KCL	: Potassium Chloride
kDa	: Kilo Dalton
LPS	: Lipopolysaccharide
MΦ	: Macrophage
MCEC-1	: Murine Cardiac Endothelial Cell one
MCP-1	: Monocyte chemoattractant protein 1
MCG	: Micrograms
MiRNAs	: Micro RNAs
mRNA	: Messenger Ribonucleic acids
Na	: Sodium
NACL	: Sodium Chloride
NADPH	: Nicotinamide adenine dinucleotide phosphate
NFKB	: Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NK	: Natural Killer cells
NKT	: Naural Killer T cells
NM	: Nano meters

NO : Nitric Oxide

NOS : Nitric Oxide Synthase

O_2 : Oxygen

OPS : Orthogonal Polarization Spectral

OSOM : Outer Stripe of the Renal Medulla

PAGE : Poly Acryl Amide Gel Electrophoresis

PBS : Phosphate Buffered Saline

PBS -/- : Phosphate Buffered Saline without Calcium or Magnesium

P13K/AKT : Phosphatidylinositide 3-kinase / protein kinase B

P38 MAPK : p38 Mitogen-Activated Protein Kinase

PG : Picograms

PI : Propidium Iodide

PMOLS : Picomoles

PPAR : Peroxisome proliferator-activated receptor

PS : Phosphatidylserine

REC A4 : Renal Endothelial Cell Line A4

REPAIR : Renal Protection Against Ischaemia Reperfusion In transplantation

ROS : Reactive Oxygen species

RRT : Renal Replacement Therapy

RT : Room Temperature

RNA : Ribonucleic acid

RTP : Room Temperature

S1P : Sphingosine 1 phosphate

S/C : Subcutaneous

SDS : Sodium Dodecyl Sulfate

sGc	: Soluble Guanylate Cyclase
SiRNA	: Short interfering RNA
TBS	: Tris Buffered Saline
TIMP-3	: Tissue Inhibitor of Metalloproteinase 3
TGF	: Tubuloglomerular Feedback
TNFα	: Tumour Necrosis Factor Alpha
UW	: University of Wisconsin
VCAM-1	: Vascular Cell Adhesion Molecule 1
VEGF	: Vascular Endothelial Cell growth Factor
ZNPPiX	: Zinc Protoporphyrin

Introduction

Background

Organ transplantation represents one of the major success stories in medicine and surgery in the 20th century. A wide variety of organs including hearts, livers, lungs, small bowel and skin can now be transplanted successfully and offer clinical benefits to recipients. Worldwide, the kidney is the most commonly transplanted organ. According to the website UKTransplant.org.uk, 1751 renal transplants were performed in the UK between 1st April 2012 and 31st March 2013[1] As of the 30th of June 2013, 7256 patients were on the waiting list for an organ transplant, 6017 of whom required a kidney[1]. The first kidney transplant in the UK took place between a pair of identical twins in Edinburgh in 1960[2]. A renal transplant remains the gold standard treatment for end stage renal failure (ESRF) which can be defined as a glomerular filtration rate (GFR) of less than 15ml per min per 1.73m^2 or a patient who is on dialysis. The UK renal registry from 2009 estimates that over 47000 people received renal replacement therapy (RRT) in the UK[3]. RRT is a supportive therapy rather than a definitive treatment for ESRF and is associated with significant complications including hypotension[4], arrythmias[5], myocardial ischaemia[6], problems of vascular access[7], pulmonary hypertension[8], depression[9] and septic complications including endocarditis[10] and oseteomyelitis[11]. Renal transplantation both prolongs life[12] and improves the quality of life for recipients[13]. It is also a more cost effective treatment than long-term hemodialysis[14]. A factsheet published by the Organ Donation and Transplantation directorate (formerly UK transplant) states that the cost benefit of transplantation compared to haemodialysis over a 10 year period (the median transplant survival time) is £24,100 per year for each year that the patient has a functioning transplant[1]. The medical and economic benefits to receiving an organ transplant are substantial. Despite proactive campaigns to improve donation rates, there remains a shortage of organs for implantation. As of March 2013 only 31% of the adult UK population are on the organ donor register[1]. Such shortages have

compelled the use of organs from "expanded criteria donors" including patients of advanced age or those with co-morbid conditions such as obesity, diabetes or hypertension. In 2012-2013 the number of deceased kidney donors increased by 11% to 1148 compared to the previous year[1]. Of these donors the proportion aged 70 years and over increased from 2 to 12%, whilst the proportion that were clinically obese (Body Mass Index of 30 and over) increased from 16 to 26% when compared to 2011-2012[1]. Organs donated from such sources are potentially of poorer quality and may negatively impact graft survival and function[15-18]. Chronic graft injury remains a significant clinical problem that is influenced by a variety of immunological and non immunological processes[19]. In the UK in 2009, 2.9% of prevalent transplant patients experienced graft failure, a figure which has remained almost constant since 2003[20]. Graft loss has major implications for patients as it necessitates either a return to dialysis or a further transplant procedure, which carries an inherent risk of morbidity and mortality. Moreover, a repeat transplant further depletes the number of donor organs available to other recipients. There is a clear need to develop appropriate strategies to address modifiable risk factors associated with graft loss. Ischaemia reperfusion injury (IRI) is associated with delayed graft function (DGF)[21], which in turn has been reported as an independent risk factor for acute rejection[22] and chronic graft loss[23]. Progress in the field of transplantation has necessitated important advances in surgical techniques and has lead to improvements in the understanding of key immunological processes. Managing organ rejection has remained an important challenge for basic scientists and transplant surgeons alike. Despite many improvements in therapies for rejection, there remains no treatment for IRI at the current time. In this thesis I shall evaluate the capacity of Heme arginate, an inducer of the enzyme Heme oxygenase-1, to ameliorate IRI within in vitro and in vivo settings.

Ischaemia Reperfusion Injury

Ischaemia Reperfusion Injury (IRI) is encountered when the blood supply to an organ or tissue is temporarily disrupted and then reinstated. IRI has been implicated in the pathogenesis of a wide range of medical and surgical conditions including trauma, myocardial infarction, sickle cell disease, ischaemic stroke, acute kidney injury, multiple organ failure and compartment syndrome [24-26]. However, the most obvious and predictable examples of IRI are to be found within the field of transplantation surgery. Donor organs become ischaemic while they are retrieved and stored prior to implantation. Following successful transplantation, restitution of the blood supply to the organ results in reperfusion injury.

Ischaemia reperfusion injury is a complex phenomenon in which tissue damage is caused by a wide range of pathological processes. IRI results in vascular leakage[27] and activation of cell death programs that result in tubular apoptosis[28] and necrosis[29]. IRI also leads to activation of the innate[30] and adaptive[31] arms of the immune system, transcriptional reprogramming[32], auto-antibody[33], complement[34] and platelet activation[35] as well as the "no-reflow" phenomenon that further may potentiate tissue ischaemia[36].

Ischaemic phase

As the name suggests, IRI is composed of an ischaemic phase that is subsequently followed by a reperfusion phase. The following biochemical changes occur in both renal tubular epithelial and endothelial cells. During the ischaemic period, aerobic metabolism is disrupted resulting in a reduction in oxidative phosphorylation. ATP synthesis falls, resulting in disturbances in cellular ion homeostasis, partly due to impaired membrane pump activity. In addition there is activation of hydrolase enzymes that causes an increase in the permeability of cellular membranes. The

intracellular pH falls as H+ ions are liberated from damaged lysosomes, in addition there is an increase in the rate of glycolysis and ATP degradation due to anaerobic metabolism. Several processes seek to regulate this change in pH and result in a number of biochemical changes that are deleterious for renal tubular cell survival. There is an increase in cytosolic Na^+ and Ca^{2+} concentrations as activity of the Na^+/K^+ adenosine triphosphatase (ATPase) falls. In addition there is increased exchange of Ca^{2+} for Na^+ by the Na^+/Ca^+ antiporter and the permeability of the plasma membrane increases. This is exacerbated by elevated cytosolic Na^+ concentrations that result in osmotic swelling leading to disruption to the plasma membrane and cell necrosis, provoking an immunological reaction [29]. Increased intracellular calcium concentrations result in the activation of hydrolases such as phospholipases (e.g phospholipase A2) and proteases that further degrade cytoskeletal proteins. Elevated plasma Ca^{2+} concentrations alter the mitochondrial membrane potential, whilst Ca^{2+} ions accumulate within the mitochondrial matrix. These changes may cause a transition pore to form within the mitochondrial membrane, allowing cytosolic ATP to gain access to the mitochondrial ATPase. This results in the further depletion of cellular ATP. During the ischaemic period, cell death occurs also occurs by apoptosis. This process is less immunostimulatory than necrosis as the cell membrane largely remains intact[37], however extracellular release of ATP from apoptotic cells through pannexin hemi-channels does occur and may attract phagocytic cells[38]. Some evidence suggests that inhibition of apoptosis may be protective murine renal IRI[39].

Tubuloglomerular feedback

One of the physiological processes by which the kidney responds to ischaemia is through tubuloglomerular feedback (TGF). Renal blood flow and GFR are maintained within relatively narrow limits by hormonal influences and by efficient autoregulation. Increases in renal blood flow cause parallel increments in GFR, thereby increasing the

necessity of tubular reabsorption and consequently increasing the metabolic and Oxygen demands of renal tissues. In the context of ischaemia, a high GFR may therefore potentiate the tissue damage sustained. Physiological coordination between renal blood flow and GFR with tubular reabsorption is maintained by the TGF system. The macular densa, a collection of densely packed epithelial cells at the junction of the thick ascending limb of the loop of Henle and the distal convoluted tubule, detects the concentration of NaCL via an apical Sodium -Potassium co-transporter. A high concentration of NaCL signifies an elevated GFR and vice versa. In response to elevated NaCL concentrations, i.e. elevated GFR, ATP is released from cells of the macular densa through pannexin channels. This extracellular ATP is converted to adenosine, which binds to Adenosine A2 receptors on extraglomerular mesangial cells, triggering a rise in intracellular calcium levels[40]. This calcium signal is then propagated via gap junctions to adjacent cells, including granular cells of the juxtaglomerular apparatus and vascular smooth muscle cells of the afferent arteriole, resulting in afferent arteriole vasoconstriction and a decrease in renin release[41]. Both of these changes tend to decrease GFR and therefore the requirement for active tubular resorption of filtered solutes, thus reducing the Oxygen requirements of renal tissues.

Reperfusion phase

During the reperfusion stage of the injury, post ischaemic kidneys are subjected to a robust inflammatory process which involves both the innate and adaptive arms of the immune response with endothelial activation, leukocyte recruitment, upregulation of chemokines and cytokines, and activation of the complement system. Reconnection of the blood supply exposes previously ischaemic tissues to increased concentrations of oxygen (O_2). This results in the generation of reactive oxygen species (ROS) as the enzymes of the mitochondrial respiratory chain which were proteolyticaly damaged

during the ischaemic period transfer electrons onto oxygen molecules. These ROS cause direct injury to cellular proteins and also result in activation of endothelial cells with upregulation of P-Selectin[42] and E-Selectin[43], and the adhesion molecules ICAM-1[44], VCAM-1[45], and PECAM-1[46]. IRI stimulates the synthesis of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α[47]. Blockade of IL-1 and IL-6 has been shown to be protective in murine renal IRI[48]. As a result of enhanced cytokine production and endothelial activation, there is increased recruitment, margination, rolling (P-Selectin) and adherence (ICAM-1, VCAM-1) of neutrophils and other leucocytes to the endothelial surface. There is also enhanced diapedesis via PECAM-1 with leucocytes crossing the endothelium and entering the tissues. These leucocytes may play a key role in IRI. Macrophages are recruited to the post capillary venules of the outer medulla following renal IRI[49, 50] and have been shown to contribute to the initiation of ischaemic acute renal failure in rats[51]. Activated macrophages may cause tissue injury via a variety of mechanisms such as the production of pro-inflammatory TNF α, induction of apoptosis, recruitment of neutrophils and elaboration of proteolytic enzymes that cause tissue injury. Indeed, evidence suggests that IRI is abrogated in animal models where macrophages are depleted[52] Other leukocytes such as neutrophils are also implicated in the pathogenesis of IRI. Anti-ICAM-1 antibody protects wild type but not neutrophil depleted mice from renal IRI[53]. The precise nature of this role is somewhat controversial however, and other authors have failed to demonstrate a protective effect of neutrophil depletion within in vivo models of renal IRI[54]. Neutrophils may be injurious by physically impeding blood flow due to their presence in increased numbers within the microvasculature or by production of harmful ROS and elastases. Other cell types that may play a deleterious role in renal IRI include natural killer

(NK) cells [55] and natural killer T (NKT) cells possibly through their capacity to recruit neutrophils[56].

Complement

The complement system plays a role in both innate and adaptive immunity in the defence against microbial pathogens. Activation of complement via the classical[57] and alternate[58] pathways has been implicated in the pathogenesis of renal IRI. The complement system may become activated in response to acute phase serum proteins such as C-reactive protein and complement mediated recognition of damaged cells, further stimulating the recruitment of inflammatory cells to sites affected by IRI[59]. Whilst studies in animals suggest that IRI may be attenuated by inhibition of specific components of the complement cascade[57, 58], clinical studies have not replicated these results[60, 61]. The complement system may in fact play a dual role in IRI, with one study of murine hepatic IRI suggesting that whilst excessive activation of the system is detrimental, a threshold level of complement activation is necessary for liver regeneration and repair[34].

Coagulation

IRI stimulates platelet aggregation[62] and the release of platelet derived mediators that further exacerbate injury[63]. Platelet activation may occur in response to integrin exposure at the basement membrane following cellular injury[64] or by transportation of platelets across epithelial barriers in association with infiltrating neutrophils[65]. The resulting microthrombi may physically impede blood flow in small vessels thereby exacerbating downstream ischaemia[66]. In addition, platelets release inorganic phosphate polymers of 60-100 phosphate residues that activate plasma protease factor XII and act in a pro-coagulant and pro-inflammatory fashion[63]. A pathophysiological role for platelets within IRI is supported by a study involving Kindlin 3 deficient mice

in a model of mesenteric IRI[64]. Kindlin 3 is a protein that plays a role in integrin mediated platelet activation. Animals in which this gene was deleted were protected against mesenteric IRI and showed virtually no platelet adherence to injured vessel walls following injury. Protection of renal function has also been associated with reduced platelet deposition in a murine model of renal IRI[67]. In this study mice were injected with HO-1 over-expressing macrophages that homed to the site of injury following IRI. Such functional protection may relate to improved renal perfusion due to carbon monoxide (a bi-product of HO-1 metabolism) related vasodilatation and inhibition of platelet aggregation. Similar mechanisms have also been implicated in clinical transplantation[68].

Implications of IRI for short and long-term graft survival

The influence of the ischaemia reperfusion injury (IRI) sustained by the organ graft at the time of surgery on long-term graft survival remains a matter of some debate[69]. IRI is established as a significant cause of acute kidney injury[70] and studies have shown a correlation between the severity of IRI at the point of organ implantation and the incidence of delayed graft function (DGF)[21, 22]. DGF is a common post-operative complication following organ transplantation. In the context of renal transplantation, DGF has traditionally been defined as a requirement for dialysis during the first post-operative week except where necessary for the management of hyperkalaemia[71]. However, more objective and functional definitions of DGF have been proposed. Functional delayed graft function (fDGF) is defined as a failure of the serum creatinine to decrease by at least 10% on 3 successive days during the first week following renal transplantation. Both traditionally defined DGF and fDGF have been shown to represent an early markers of subsequent inferior allograft outcomes[71, 72]. DGF is particularly associated with elderly[73], marginal[74, 75] and cadaveric organ donors[76]. The incidence of delayed graft function varies between centres[22], but has

been shown to affect between 8 and 50% of organ transplants in the USA[23, 77]. Delayed graft function itself is a significant clinical problem that is associated with a prolonged hospital stay[15], increased economic costs[78] and a requirement for further dialysis in some cases[21]. Moreover, DGF may have implications for long-term outcomes following organ transplantation. Ojo et al have shown DGF to be an independent negative prognostic factor for renal allograft survival at 5 years[23]. In this study, significantly fewer allografts with DGF but no evidence of acute rejection survived to 60 months post transplant compared with grafts that exhibited neither acute rejection nor DGF. However, the mechanism underlying this association and the relative contribution of immune priming or injuries to the tubular and endothelial compartments remains uncharacterised. Requiao-Moura et al[22] evaluated a group of 628 patients that underwent deceased donor renal transplantation in Brazil between 2002 and 2005. These authors found a correlation between DGF and acute rejection events as well worse graft function and lower graft survival rate at one year. Other authors have also shown DGF to be associated with an increased risk of acute rejection and reduced graft function and survival after one year[21]. However, some studies question whether a direct link exists between delayed graft function and subsequent chronic graft dysfunction. Boom et al[79] found DGF to be associated with a prolonged cold ischaemia time in a study of 734 cadaveric renal transplants performed between 1983 and 1987. These authors also noted an association between DGF and both acute rejection and suboptimal function at one year, but, in contrast to the findings of Ojo et al, found no independent association between DGF and chronic graft loss. Work by Troppmann et al suggested that whilst DGF in isolation has no direct association with late graft failure[80], DGF when combined with acute rejection at one year was associated with late graft loss[81]. The link between DGF and acute rejection at one year appears to be consistently established across these studies, as is an association between acute rejection and chronic graft loss. Whilst the independence

of DGF as a risk factor for late graft loss is a matter for debate, there is agreement about the association between the severity of IRI and DGF. Interest has focussed upon strategies that seek to limit the severity of IRI following transplantation as a means of improving the short and long-term outcomes following solid organ transplantation.

Current Strategies to limit Ischaemia Reperfusion Injury

The introduction of new and highly effective immunosuppressive therapies has resulted in a dramatic reduction in acute rejection rates in recent years; however, whilst a number of measures seek to minimize the impact of IRI, no specific treatments for the phenomenon are currently available. Several strategies have achieved some success in minimizing the extent of the injury caused by IRI and these are briefly summarised below.

Ex vivo normothermic perfusion

Ex vivo normothermic perfusion (EVNP) is an emerging technique that attempts to improve the outcome following solid organ transplantation by minimizing IRI. EVNP involves the active, mechanical perfusion of individal organs ex vivo with preservation solutions maintained at normal body temperatures prior to implantation. This is in contrast to static cold perfusion in which organs are stored in cold preservation solutions (i.e in immobile preservation fluid, without mechanical perfusion). The technique has been shown to result in improved renal function and lower levels of tubular injury when compared with static cold stored kidneys in a porcine model of renal transplantation[82]. Organs that received EVNP in this study showed reduced expression of the inflammatory cytokines TNFα, IL-1β and IL-8 but increased expression of IL-6 when compared with the static cold stored organ group. The technique has undergone clinical trials in human transplant patients where it has been reported to be safe and to significantly reduce the rate of DGF in marginal

kidney donors[83] Organs treated with EVNP have been shown experimentally to have higher ATP levels when compared to cold stored organs, which may potentially explain the beneficial effects of the technique[84]

Preservation solutions

Organ preservation solutions were developed for the storage of organs prior to implantation. Examples of such solutions include Viaspan, also known as University of Wisconsin (UW) solution, histidine-tryptophan-ketoglutarate (HTK) solution and Celsior solution. UW solution contains a variety of substances that aim to counteract various elements of the injurious metabolic cascade associated with IRI. The solution was developed to maintain an osmotic concentration by the use of metabolically inert substances like lactobionate and raffinose rather than glucose. UW solution also contains Hydroxyethyl starch to prevent oedema during the ischaemic phase of IRI and free radical scavengers such as Glutathione to counter the generation of ROS produced upon organ reperfusion. In addition, UW solution contains Monopotassium phosphate, Magnesium sulphate, adenosine and Allopurinol. UW has been reported to increase the time period for which organs such as liver, kidney and pancreas can be stored prior to implantation[85]. The effectiveness of UW solution has been examined in a number of clinical and laboratory based trials and has been found to have a similar safety and efficacy profile to Celsior solution[86, 87]. UW is used as the storage medium for organs in the model of murine renal isograft transplantation employed in this thesis.

Ischaemic and remote preconditioning

Other strategies aim to increase an organ's tolerance to ischaemia in order to limit the effects of IRI. This may be achieved by deliberately exposing tissues to multiple, shortened periods of ischaemia followed by reperfusion prior to subsequent

transplantation or exposure to a more prolonged period of ischaemia. Such measures are termed "ischaemic preconditioning". Clinical trials of ischaemic preconditioning in human patients have shown some promising results in liver transplantation[88] and in liver resection[89], although the protective benefits observed in these studies are not as striking as those seen in animal models. The underlying protective mechanisms behind ischaemic preconditioning are thought to be multi-factorial, but may involve the induction of the enzyme Heme oxygenase-1 (HO-1)[90, 91]. HO-1 is an enzyme that catabolises Haem rings to Carbon Monoxide (CO), Biliverdin and free Iron. Interestingly some studies demonstrate protection even when the organ itself is not directly exposed to periods of ischaemia. Subjecting the hind limbs of rats to periods of intermittent ischaemia has been shown to confer hepatic protection in a model of liver IRI[92]. This strategy has been termed "remote ischaemic conditioning". HO-1 has again been implicated in the mechanism of this protection[93]. Remote Ischaemic preconditioning has been shown to reduce hepatocellular injury in animals undergoing hepatic IRI, and to improve sinusoidal blood flow by increasing sinusoidal diameter[94]. A potential explanation for this vasodilatation may be increased carbon monoxide production as a bi-product of enhanced HO-1 activity. Ischaemic preconditioning has been trialled in human patients undergoing major liver resection. It was shown to protect against post-operative liver injury in non cirrhotic patients[89]. Remote ischaemic preconditioning has been evaluated as an additional therapy prior to percutaneous coronary artery angioplasty. The technique increased myocardial salvage following angioplasty[95]. A form of remote ischaemic conditioning has entered clinical trials in human patients. The REPAIR trial (Renal Protection Against Ischaemia-Reperfusion in transplantation) is currently underway in 7 transplant centres in the UK. Investigators are assessing a group of 406 living donor renal transplant patients. Both the donor and recipient are randomised to receive either 3 cycles of 5 minutes of inflation of a blood pressure sphygmomanometer cuff on their

upper arm followed by 5 minutes of rest or a placebo treatment. This intervention takes place 24 hours prior to transplant surgery. The primary objectives of the study will be to assess whether this has an impact on GFR 12 months after transplantation. Secondary objectives will be, amongst others, to study whether the intervention affects the rate of fall in creatinine in the first 72 hours after transplantation, the inflammatory response to surgery in the first 5 post-operative days, kidney fibrosis 6 months after trasnplantation and patient outcomes 2-5 years after organ transplantation using renal registry data. At the time of writing the results are unknown.

Pharmacological strategies

A more common focus of experimentation has been the use pharmacological agents to ameliorate IRI. A variety of drug treatments have been used with the intent of influencing the various pathways that are implicated in IRI, and these pre-treatment strategies have achieved differing degrees of success in animal models[96-98] Unfortunately however, no treatment has as yet been shown to reduce mortality significantly in human patients. This may reflect the fact that the aetiology of acute kidney injury (AKI) is multi-factorial, complex and incompletely understood[99]. Clearly, although the mechanisms of IRI responsible for AKI may be similar to those involved in organ transplantation, the clinical context in which the IRI occurs in these two scenarios differs. Transplantation is a planned, predictable event and therefore provides greater possibilities for the use of pharmacological "preconditioning" strategies that aim to reduce the damage sustained following subsequent IRI, in contrast to "treatment strategies" that seek to ameliorate an established injury. I shall discuss pharmacological preconditioning strategies in a subsequent section. Broadly speaking, "treatment strategies" tend to fall into one or more main categories, reflecting the underlying pathophysiology of IRI.

Antioxidants and ROS scavengers

A range of antioxidant molecules have been utilised in an attempt to reduce the cellular and protein damage induced by ROS. Lee et al[100] have demonstrated that the antioxidant ascorbic acid (vitamin C), administered following surgery, attenuated structural and functional injury in a canine model of renal auto-transplantation. Other authors have noted protective benefits in rat models of renal IRI following treatment with the free radical scavenging agent Edaravone[101], the inducible nitric oxide synthase (iNOS) inhibitor Aminoguanidine[102] and the amino acid Taurine (which has antioxidant activity)[103]. Of interest, two clinically licensed anti hypertensive drugs, Cavedilol[104, 105] and the angiotensin-converting enzyme (ACE) inhibitor zofenopril[106] have been shown to attenuate renal IRI in animal models, whilst reducing measures of oxidant activity. Despite their recognized side effects, such drugs have the potential to translate into practical therapies. Unfortunately no clinical trials have yet reported benefits of similar therapeutic strategies in human patients. Although a preliminary report has suggested early benefits of antioxidant supplementation in a small case series of renal transplants[107], other studies have demonstrated a lack of benefit from such agents in the long-term[108].

Inhibitors of apoptosis

Reduction in the amount of cell death, through inhibition of apoptosis, has also been proposed as a therapeutic strategy in renal IRI and a variety of drugs have been investigated in animal models. Caspases are a family of cysteine proteases that play essential roles in apoptosis, necrosis and inflammation[109]. Caspase inhibitors are effective in reducing renal injury in animal models of IRI[110, 111] and pancaspase inhibitors have entered early clinical trials in human patients[112]. Minocycline, a tetracycline antibiotic which inhibits apoptosis via attenuation of TNFα has been shown to reduce tubular cell apoptosis[113], renal inflammation[114] and microvascular

permeability[114] in rat models of renal IRI. Other potentially therapeutic anti apoptotic agents include poly ADP-ribose polymerase (PARP) inhibitors[115] and p53 inhibitors such as Pifithrin-α[116]. Activated protein C (APC), an anticoagulant generated by the thrombin-thrombomodulin complex in endothelial cells, has been shown to have an anti apoptotic and anti-inflammatory effect[117, 118]. It has been demonstrated that APC reduces renal IRI through inhibition of leukocyte activation[119].

Growth factors

Erythropoietin (EPO) is a glycoprotein hormone that promotes erythropoiesis. Exogenous administration of EPO has been shown to attenuate AKI in rats by reducing tubular apoptosis and necrosis[120, 121]. EPO also promotes the mobilisation and proliferation of endothelial progenitor cells from the bone marrow and these cells have been shown to participate in tissue repair[122, 123]. Recombinant EPO is licensed and used in clinical practice, meaning that such findings have some potential to become practical therapies.

Endothelin-1 antagonists

Endothelin-1 (ET-1) is a potent vasoconstrictor that is upregulated in conditions of ischaemia[124] and has been implicated in the pathogenesis and potentiation of renal IRI[125]. ET-1 may also play a pro-inflammatory role in renal IRI by stimulating the upregulation of adhesion molecules and the production of cytokines from leukocytes[126]. The endothelin receptor antagonist, tezosentan, has been shown to protect renal function in a rat model of renal IRI[127]. ET-1 inhibition may represent an appropriate target for pharmacological interventions aimed at ameliorating renal IRI.

Anti-inflammatory drugs

Leukocytes including neutrophils, monocytes, macrophages and T cells play important roles in renal IRI, and several drugs have shown potential to ameliorate renal IRI through an anti-inflammatory action. Many of these agents are already licensed in human patients and are well tolerated, increasing their potential for translation into practical therapies. Such drugs include sphingosine 1-phosphate (S1P) analogs such as FTY720. S1P is a ligand for a family of G protein coupled endothelial differentiation gene receptors that regulate a diverse range of cellular signalling processes including cell survival, apoptosis, cell adhesion and movement[128]. FTY720 acts as a S1P agonist, leading to sequestration of lymphocytes in secondary lymphatic tissue[129]. FTY720 produced lymphopenia and resulted in tissue protection in a murine model of renal IRI[130]. Selective adenosine agonists have also been proven to be protective in animal models of renal IRI. Adenosine normally binds to members of the G protein-coupled receptor family and regulates a wide variety of physiological processes including elaboration of ROS by neutrophils and leukocyte adherence to endothelial cells[131]. The selective A2A agonist ATL146e is highly effective against renal IRI in rodent models[132]. Other authors have demonstrated that pre-treatment of mice with the steroid dexamethasone can prevent structural and functional damage in a model of ischaemia reperfusion injury[133]. This protection was associated with attenuation of inflammation. Mechanistic studies confirmed that dexamethasone promoted activation of the glucocorticoid receptor in this model by which it attenuates P13K/AKT activation and consequently the inflammatory response. Infliximab, a chimeric monoclonal antibody against TNF α, is commonly used in the clinical management of autoimmune diseases, such as Crohns disease, ulcerative colitis and rheumatoid arthritis. Administraion of Infliximab prior to reperfusion has been shown to reduce both structural injury and inflammation in a rat model of IRI[134].

Other classes of drugs with potential to ameliorate renal IRI include inducible nitric oxide synthase inhibitors[135] and fibrates which bind to peroxisome proliferator-activated receptors (PPAR), suppress NF-kB activation, chemokine expression and neutrophil infiltration[136].

The Heme oxygenase system

The heme oxygenase enzymes catalyse the degradation of heme molecules into carbon monoxide (CO), biliverdin and free iron (Fe^{2+}). Heme oxygenase exists as three distinct isoforms in human beings, Heme oxygenase-1, 2 and 3 (HO-1, 2 and 3). HO-1 and HO-2 are the most prevalent forms of the enzyme. HO-2 has a molecular weight of 36kDa, and is expressed constitutively in the liver, Leydig cells of the testis[137] and kidney[138]. Recent evidence suggests that HO-2 may be inducible to a certain extent in myocardium in response to prolonged periods of hypoxaemia[139]. HO-2 is thought to play an essential role in maintaining renal haemodynamics and function[138]. HO-2 deficiency has been associated with major renal morphological injury and impaired renal function in diabetic mice[140]. The final isoform to be discovered, HO-3, was characterised in the 1990s. McCoubrey et al reported the presence in rats of a 2.4kb transcript encoding a 33kDa protein[141]. HO-3 transcript was detected in a series of organs including spleen, liver, kidney and brain[141]. HO-3 remains the most elusive and poorly understood HO isoform. Although HO-3 mRNA is reported to be present in rat tissues[141, 142], its protein expression has not been detected at tissue level. Work by Hayashi et al[143] suggested the presence of two HO-3 pseudogenes in rats, tentatively named HO-3a and HO-3b. These authors concluded that these may be derived from HO-2 transcripts and that they were not associated with expression of a functionally active protein.

The first of the Heme oxygenase isoforms to be characterised and the most extensively studied is HO-1. Tenhunen et al[144] in 1968 were the first investigators to present evidence of a previously undescribed enzyme system in microsomes capable of degrading heme to bilirubin. The enzyme has generated much interest over recent years following reports of its cytoprotective capacity[145]. Evidence has accumulated that HO-1 may play a role in a number of disease states. Some studies have suggested that GT repeat polymorphisms within the gene's promoter region may increase the quantitative level of HO-1 activity in response to a given stimulus. This in turn may be linked to a more favourable prognosis in a number of clinical conditions including abdominal aortic aneurysm[146], ischaemic cerebrovascular events[147], hypertension[148] and ischaemic heart disease[149]. Specifically, such polymorphisms have been associated with improved outcome following renal transplantation[150, 151], but this has not been reproduced in all population studies[152, 153]. Diminished levels of HO-1 expression may also be pathological. Kawashima et al[154] report an autopsy examination performed on a six year old boy with the only recorded case of HO-1 deficiency. The boy was born to parents each of whom had diminished HO-1 levels. He presented clinically with signs of growth retardation, anaemia, leukocytosis, thrombocytosis, coagulopathy, elevated serum haptoglobulin, ferritin and heme in serum. A low serum bilirubin and hyperlipidaemia were also noted. At autopsy, amyloid deposits were identified in the liver and adrenal glands. Fatty streaks and fibrous plaques were also present in the aorta, an unusual finding in such a young patient. There are some similarities between these findings and the phenotype of HO-1 knockout mice. However, the human case seemed to disproportionately involve endothelial cells and the reticuloendothelial system, resulting in intravascular haemolysis, disseminated intravascular coagulation and amyloidosis. By contrast, HO-1 deficient mice survive for relatively longer periods and are predominantly affected by disorders of iron metabolism[155].

HO-1 is a 32kDa enzyme encoded by the HMOX-1 gene that is located on the long arm of chromosome 22 at position 13.1[156]. The gene contains 5 exon regions and spans approximately 14kb. Work by Alam et al[157, 158] has identified the presence of two promoter regions that confer responsiveness to most inducers in the mouse HO-1 gene. These regions have been termed E1 and E2 (enhancer region 1 and 2 respectively). The mouse HMOX-1 gene is thought to share a high degree of homology with the human form of the gene, and regions similar to these have been found within the human HO-1 promoter[159, 160]. A further enhancer region within the human HO-1 gene itself is also involved in heme and cadmium mediated HO-1 induction[161]. HO-1 is induced by a wide variety of stimuli, a common characteristic of these inducers being their capacity to cause oxidative stress [160, 162-164]. HO-1 catalyses the breakdown of heme rings at the α-methene bridge to liberate carbon monoxide (CO), free iron (Fe^{2+}) and biliverdin, the latter is subsequently reduced by biliverdin reductase to bilirubin. Rapid metabolism by HO-1 of the heme molecules produced following IRI prevents the generation of harmful reactive oxygen species via the Fenton reaction[165]. Pharmacological induction of HO-1 or substitution with the end products of the reaction catalysed by HO-1 has shown some potential to be protective in animal models of IRI[166, 167] However, other studies using metal protoporphyrins as HO-1 inducing agents have demonstrated a worsening injury[168]. Preconditioning strategies that utilise pharmacological inducers to upregulate HO-1 prior to organ implantation have been reported to protect in animal models of hepatic[169], renal[170] and cardiac transplantation[171]. Such data has stimulated interest in a potential role for HO-1 in ameliorating transplant-associated IRI in human patients. At the time of writing, no clinical trials have been undertaken using HO-1 inducing agents in human transplant patients. The majority of animal studies published so far utilise heavy metal protoporphyrins as HO-1 inducing agents. Such reagents are not licensed for use in

human patients, limiting their capacity for such work to form the basis of practical therapies.

The therapeutic benefits of HO-1 induction are mediated through the end products of the reaction that it catalyses. Each of these substances impacts on a variety of injurious pathways and mediators associated with IRI. This has led some authors to speculate whether the heme oxygenase system may represent an evolutionarily conditioned defence mechanism in human beings[172]. Carbon monoxide (CO) promotes vasodilatation by acting through guanyl cyclase/cGMP and activation of potassium activated cellular channels[173]. It is also anti-apoptotic in endothelial cells and leukocytes through activation of p38 MAPK[174], and attenuates ICAM –1 expression by inhibiting NFKB. CO derived from enhanced HO-1 activity inhibits the activity of NADPH oxidase thereby suppressing the overproduction of superoxide radicals (O2-) and the accumulation of reactive oxygen species in lipopolysaccharide stimulated macrophages. Bilirubin is a potent anti-oxidant, countering the deleterious action of reactive oxygen species generated during ischaemia. Free iron generated by HO-1 activity is rapidly removed through post-transcriptional upregulation of ferritin. This has the additional benefit of sequestering other Fe^{2+} ions in the milieu preventing the production of harmful ROS via the Fenton reaction. The Fe^{2+} liberated by HO-1 also activates the ATPase Fe^{2+} secreting pump, which decreases the intracellular free Fe^{2+} content. Importantly, bilirubin and the depletion of free iron have been shown to inhibit the ability of pro-inflammatory cytokines such as TNFα and IL-1β to induce the expression of the adhesion molecules E-selectin, P-Selectin, ICAM-1 and VCAM-1 via inhibition of NF kappa B[175]. These adhesion molecules are associated with endothelial activation and facilitate the binding of leucocytes to the endothelium, where their harmful effects are exerted.

A wide range of stimuli and clinical conditions result in increased HO-1 expression including transplantation itself[176]. However, it seems likely that this increase is either too little or too late to afford maximal protection against the severity of ischaemia reperfusion injury encountered in transplantation. Strategies aim to induce over-expression of HO-1 to prevent transplant associated injury work by "preconditioning" tissue such that HO-1 is already increased at the time-point at which the ischaemia reperfusion injury occurs. Cells and tissues within the transplanted organ are likely to be particularly vulnerable to injury in these initial stages, when they produce insufficient HO-1 to counter the ischaemia reperfusion injury to which they are exposed.

Heme arginate

A growing body of literature suggests that over-expression of HO-1 may be protective in animal models of IRI[168, 169, 177, 178] and transplantation[170, 171, 179]. As mentioned previously, the majority of such studies utilise metal protoporphryins as HO-1 inducing agents. These substances have numerous toxic side effects and are unstable at physiological pH, making them unsuitable for use in humans. Heme arginate (HA, Normosang TM, Orphan Europe) is a clinical grade heme protoporphyrin licensed in the treatment of porphyria[180, 181]. The drug is generally well tolerated clinically, although a case report of anaphylaxis attributed to HA has been published[182]. The British National Formulary lists the principal side effects of HA as fever and thrombophlebitis at the injection site. A recent clinical study has demonstrated that HA treatment upregulates HO-1 in healthy human volunteers, providing further validation for its potential use in clinical trials[183]. In vivo studies that utilise HA are scarce but appear to offer further evidence of the protective benefits of this drug. HA improved liver microcirculation and mediated an anti-inflammatory cytokine response in a rat model of simulated haemorrhagic shock[184]. Work from within our own group

has shown that HA pre-treatment reduces structural injury and preserves renal function in aged mice subjected to renal IRI[50]. In this thesis I shall evaluate the capacity of HA to upregulate HO-1 and ameliorate IRI within in vitro and in vivo models. Given its favourable safety profile and capacity to induce HO-1 in humans, HA treatment has clear potential to translate into a practical clinical therapy.

Donor vs. recipient preconditioning strategies

The majority of studies that use preconditioning agents in models of transplantation concentrate on treatment of the organ donor, rather than the organ recipient. Translating this work into human patients poses a number of practical and ethical considerations[185]. Although the pre-treatment of brain dead organ donors is already well established with regard to thyroid hormones[186] and metyhlprednisolone[187], these therapies function to stabilize the organ donor's clinical condition and correct the endocrine abnormalities that occur after brain stem death. By contrast, administration of an agent to the organ donor with the sole intent of optimizing that donor's organs for transplantation is deemed less ethically acceptable. Any preconditioning measure employed in such a manner may also have implications for the functioning of the donor's other organs and may influence their suitability for transplantation. Systemic treatment of the organ donor may not be practical unless it can be demonstrated that such measures at least do not adversely affect the outcome following transplantation of the other organs. Such practical considerations may be negated by preservation techniques such as EVNP, where the organ transplant is treated in isolation Ex vivo[83]. An acceptable alternate strategy may be to administer preconditioning agents to organ transplant recipients. This would negate those issues associated with donor pre-treatment. Moreover, there may be additional benefits to be derived from systemic treatment; for example, the immune responses within the recipient to the transplanted organ may be modified, with potential effects on both acute and chronic allograft

rejection. Recipient preconditioning, although not extensively studied in the literature, has been shown to be effective in animal models of cardiac[171] and renal transplantation[179]. The over-expression of heme oxygenase-1 (HO-1) has been reported to confer therapeutic benefits in animal models of IRI and transplantation. One study in transgenic mice demonstrated improved allograft survival rates in recipient mice that systemically over-expressed HO-1 when compared with normal mice that received a graft from a donor manipulated to over express HO-1[171]. Some of this additional benefit may relate to upregulation of HO-1 in the recipient animal's circulating leukocytes, which subsequently infiltrate the graft and consequently target therapeutic molecules directly to the site of tissue injury. Clearly, it is essential to understand whether donor or recipient treatment is the most effective way to employ a preconditioning strategy.

The endothelium and microvascular dysfunction following IRI

Preservation of an intact renal microvasculature during IRI is emerging as a key strategy in prevention of transplant associated injury[188]. IRI causes profound disruption to the microvasculature, resulting in microvascular leak[27] and the "no reflow" phenomenon in which downstream tissue perfusion remains impaired despite reconnection of the blood supply, due to physical obstruction of the lumina of small vessels by thrombus, cell debris, leukocytes or by diminished vasodilatation[36]. The endothelium is more than a physical barrier between the vascular system and interstitium. It forms a dynamic interface regulating haemodynamic, inflammatory and anti-thrombogenic processes. IRI results in endothelial dysfunction by inhibiting the capacity of the endothelium to regulate these processes. The endothelium elaborates a variety of substances to maintain vascular homeostasis. Endothelial cells produce nitric oxide via endothelial nitric oxide synthase. Nitric oxide promotes vasodilatation and prevents the upregulation of endothelin-1, a potent vasoconstrictor

induced in ischaemic conditions[124]. NO also prevents leukocyte adherence to the endothelium that can further compromise blood flow[189]. Nitric oxide produced by endothelial cells inhibits platelet aggregation and cytokine induced expression of tissue factor[190]. In addition, hypoxia also reduces the expression of the anticoagulant thrombomodulin by endothelial cells[191] and promotes the expression of a direct activator of Factor X[192]. A damaged endothelium, subjected to hypoxic conditions, therefore promotes thrombosis, which further impairs the blood supply to the distal tissues. Loss of NO production leads to an increase in the levels of the vasoconstrictor endothelin-1 and also diminishes the capacity of the microvasculature for vasodilatation by NO-mediated mechanisms. These changes predispose the graft to further ischaemic damage and contribute to the "no-reflow" phenomenon. In addition, IRI increases vascular permeability by disrupting the physical integrity of the renal vascular endothelium[193, 194]. Studies using cultured endothelial cells exposed to hypoxia have demonstrated an increase in cell permeability secondary to lower levels of cAMP[27, 192]. Further evidence of increased microvascular permeability is observed in mice subjected to prolonged hypoxia, with pulmonary oedema and albumin leakage into multiple organ systems[195, 196]. In addition, T cells may enhance vascular permeability in ischaemic renal failure[197]. The hypothesis that endothelial cell dysfunction may mediate the haemodynamic consequences associated with IRI is further supported by a study that showed that renal function was improved in a rat model of renal IRI by the transfer of viable endothelial cells. Brodsky et al[198] used minimally invasive intravital microscopy to provide direct evidence of the "no reflow" phenomenon within the peri-tubular capillary network of rats subjected to renal IRI which was attributable, in part, to endothelial injury. Implantation of endothelial cells, or surrogate cells expressing nitric oxide synthase resulted in dramatic functional protection of ischaemic kidneys. The authors conclude that endothelial cell dysfunction is the primary cause of the "no-reflow" phenomenon.

Recent advancements in imaging technology have enabled direct visualisation and analysis of the microcirculation in the immediate aftermath of IRI. Using a rat model of kidney transplantation Hölzen et al[199] showed that donor preconditioning with the HO-1 inducer hemin led to significantly lower serum creatinine and less histological damage compared with control animals. In vivo microscopy of the renal surface one hour following reperfusion showed significant enlargement in the vascular diameter and an increase in the capillary flow in the hemin treated animals. This may relate to the vasodilatory effects of CO. Andonian et al[200] analysed the microcirculation in a murine model of renal IRI in real time by using targeted microbubbles. These authors determined that blood flow to the kidney decreased from 554m/s to 182m/s following simulated renal IRI. The corticomedullary junction, which had the highest rate of blood flow in animals not subjected to IRI, was the site of the greatest upregulation of P-selectin following IRI (41% increase compared to increases of 25% in the cortex and 14% in the medulla). P-selectin is an adhesion molecule that is expressed on endothelial cells and activated platelets and is an established marker of inflammation and ischaemic injury[201, 202], moreover P-selectin facilitates the binding of injurious leukocytes to the endothelial surface. Administration of anti-P selectin monoclonal antibody has been shown to attenuate IRI[202]. The authors conclude that the corticomedullary junction sustains the highest degree of nephron and microvascular damage and is most susceptible to ischaemic injury. Such findings correlate with the model of native kidney IRI used in this thesis, in which the corticomedullary junction sustains the greatest degree of acute tubular necrosis[50] and is the site of greatest platelet deposition[67].

Evidence of a direct link between endothelial dysfunction and adverse clinical outcomes following renal transplantation in human patients is difficult to obtain.

Schmitz et al[66] used non-invasive orthogonal polarization spectral (OPS) imaging to visualize and quantify cortical kidney microcirculation in 13 combined kidney/pancreas recipients. Upon reintroduction of the blood supply, a heterogeneous pattern of perfusion was observed. There was oscillating flow and scattered microvascular thrombosis of peritubular capillaries, resembling 'no reflow'. Volumetric capillary blood flow showed a significant positive correlation with the measured decrease in creatinine between days 1 and 3, linking early microvascular changes with clinical parameters. The authors conclude that such a technique may have a future role in predicting early ischaemia reperfusion injury induced graft dysfunction. In conjunction with the work of Ojo et al[23], which linked the severity of initial IRI with DGF and consequent impaired survival of renal allografts at 5 years, this suggests that early endothelial dysfunction may play a role in chronic graft loss. The study of the endothelium in transplanted organs of living transplant patients by invasive procedures such as multiple renal biopsies is difficult to justify for purely research purposes. Indirect, non-invasive techniques such as high-resolution brachial artery ultrasound have been reported to be efficient measures of endothelial dysfunction[203]. However, such analyses may be complicated by the fact that endothelial dysfunction is known to be more commonplace in patients with ESRF undergoing haemodialysis; such patients are also subject to increased cardiovascular morbidity and mortality[204, 205]. Moreover, a functioning renal transplant corrects metabolic abnormalities and normalises endothelial dysfunction[206, 207]. The analysis of circulating endothelial cells (CEC's) may provide indirect evidence of an association between microvascular dysfunction and graft injury in transplant recipients. Such cells become detached from the basement membrane as a consequence of an injurious process. Work by Woywodt et al[208, 209] measured the numbers of CEC's in the blood of 129 renal transplant recipients who underwent percutaneous graft biopsy at 1 to 245 months (mean 37 months) following transplantation. The authors reported higher

levels of CEC's in transplant recipient patients than controls regardless of their biopsy findings. On biopsy, seven patients had acute vascular rejection, 15 patients had acute tubulointerstitial rejection and 93 patients had no rejection. Patients with acute vascular rejection had the highest cell numbers when compared with all other patients. Mohammed et al[210] have also shown a correlation between CEC number and allograft rejection. These authors conclude that circulating endothelial cells may be a novel marker of endothelial injury and endothelial dysfunction. The correlation between rejection episodes and CEC number also suggests an association between endothelial injury or dysfunction, and adverse outcome following renal transplantation.

Macrophages

Tissue resident macrophages (derived from the Greek macros: large and phagein: eat) are leucocytes of myeloid lineage. Macrophages are key players in both the innate and adaptive arms of the immune system. They are derived from circulating monocytes that exit the circulation via the process of extravasation to become macrophages when they enter the tissue spaces. This process may occur in response to cytokines released as part of the inflammatory process by pathogens, dying cells or other macrophages. In addition to macrophages that are "recruited" from the circulation, tissue resident macrophages are also found in a number of organ systems under normal circumstances. Examples include macrophages situated in the liver (Kupffer cells), brain (microglial cells), bone (osteoclasts) and lungs (alveolar macrophages). Macrophages are capable of a diverse and complex range of functions[211]. A macrophage's phenotype depends upon on its activation status, which is influenced by a variety of factors including the milieu in which the cells reside and their interactions with other cell types[212, 213]. As a consequence, macrophages may be influenced to behave in a "pro-inflammatory" or "pro-repair" fashion in differing situations. These states have traditionally been termed "classically activated" (M1) and "alternatively

activated" (M2). Microbial products and cytokines such as IFNγ induce macrophages to adopt an M1 phenotype. M1 macrophages are considered to be "pro-inflammatory" and are microbicidal through their production of ROS, NO and lysosomal enzymes. They also produce chemokines and interleukins such as IL-1, IL-12 and IL-23, which participate in the inflammatory cascade. M2 macrophages by contrast play important roles in tissue repair, the defence against helminthic parasites and in the resolution of inflammation. M2 macrophages are induced by cytokines, helminths and interleukins IL-13 and IL-4. Alternatively activated macrophages elaborate IL-10 and TGF-β that dampen down inflammatory responses, they also produce Arginase, proline and polyaminases that are involved in wound repair and fibrosis. The separation of macrophage phenotypes into "pro-inflammatory" and "pro-repair" groupings is somewhat of an over simplification. It has become increasingly apparent in recent years that no clear binary distinction exists between M1 and M2 states, rather, there is a continuum between the two extremes[214].

Therapeutic potential of macrophages

It is apparent that macrophages may function in a deleterious or beneficial manner depending on their microenvironment and the various factors to which they are exposed. Interest has focussed upon the potential to manipulate macrophage phenotype toward the "anti-inflammatory" end of the spectrum in an attempt to modify the outcome in a variety of disease processes in animal models. Macrophages obtained from experimental animals may be virally transduced, stimulated by cytokines or subjected to drug treatment to alter their phenotype ex-vivo prior to re-injection to the animal model as a form of "cell based therapy". In a study using a rat model of nephrotoxic nephritis, alveolar macrophages were transfected with adenovirus to induce the expression of the anti-inflammatory cytokine IL-4 prior to

re-injection into the renal artery [215]. The transformed macrophages localised to glomeruli and were shown to express IL-4 in-vivo. Treated animals were found to have reduced levels of albuminuria. Histological markers of glomerular inflammation and macrophage infiltration were also diminished. Interestingly, a protective effect was also demonstrated in the contra-lateral, non-injected kidney. This suggests that such treatments may have effects beyond the mere physical association of altered macrophages with the structures intended for protection. Potential explanations may include a more generalised alteration of inflammatory responses at the systemic level by the modified macrophages. The practicality of translating such techniques into clinical therapies remains unclear, however, it raises pertinent questions as to the most effective way of delivering therapeutic benefits from preconditioning strategies that might depend in part upon macrophage phenotype, and is especially relevant in the context of organ transplantation. Organ donor treatment may alter the phenotype of macrophages resident in the transplanted organ, such that they are present at the time when IRI occurs. They are therefore capable of providing protective benefits in the immediate aftermath of IRI at which time tissues are most vulnerable to injury. Treatment of the organ recipient, however, might favourably manipulate systemic inflammatory responses following organ transplantation. Furthermore, recipient treatment may affect circulating monocytes within the recipient and thereby facilitate greater recruitment of altered, therapeutic macrophages from the circulation to the site of injury. In the context of transplantation, where there is inherent physical disassociation of organs from donor to recipient, it will be important to accurately determine which cells are important in affording any protection against injury. Determining whether HO-1-positive macrophages have a protective role in the context of organ transplantation is of interest not only on a scientific basis, but may also have implications for how preconditioning treatments may be employed

clinically and is of particular relevance given the practical concerns surrounding organ donor treatment discussed earlier.

The role of macrophages within IRI and transplant systems

Evidence suggests that macrophages play a key role in propagating injury within animal models of hepatic[216, 217] and renal IRI[218]. It has also been demonstrated that macrophages cause destruction of the renal microvasculature in murine kidney transplantation[219]. Several potential mechanisms may be responsible for these effects. During the relative hypoxia of ischaemia reperfusion injury, macrophages generate reactive oxygen species that cause direct injury to the transplanted organ, or may "activate" the endothelium by inducing the upregulation of cellular adhesion molecules such as ICAM-1 and VCAM-1[46, 216, 217]. This activation of the endothelium primes the transplanted organ for direct injury by neutrophils during the subsequent reperfusion phase of the injury[220].

Effect of HO-1 induction upon macrophage phenotype in IRI

Manipulation of macrophage phenotype may therefore offer potential to ameliorate IRI and emerging evidence suggests that HO-1 induction may play a key role in this. Induction of HO-1 protein within bone marrow derived macrophages (BMDM) has been shown to confer an anti-inflammatory phenotype. Ferenbach et al[67] reported reduced production of TNFα and nitric oxide combined with increased levels of IL-10 and increased phagocytosis of apoptotic cells in BMDMs that were virally transduced to overexpress HO-1 prior to stimulation with IFNγ and LPS. Such work echoes the findings of Otterbein et al who demonstrated that the carbon monoxide generated by the activity of heme oxygenase -1 may have anti-inflammatory effects upon macrophages[221]. These authors demonstrated both in vivo and in vitro that carbon

monoxide at low concentrations inhibited the expression of the LPS induced pro-inflammatory cytokines tumor necrosis factor-α, interleukin-1β and macrophage inflammatory protein-1β. CO also increased the expression of the anti-inflammatory cytokine interleukin-10. Another study demonstrated that inhibition of HO-1 during culture of BMDMs resulted in adoption of a pro-inflammatory Ly6c+, CD11c-, F4/80- phenotype[222]. It should be noted that, given their inherent complexity and capacity to be influenced by a range of stimuli, it might be difficult to extrapolate from in vitro experiments how a macrophage may function within the ever-changing environment of an in vivo system. More compelling evidence as to the importance of HO-1 positive macrophages in IRI comes from studies in which such cells are added or depleted within in vivo models. Kupffer cells have been reported to be the major source of hepatic HO-1 expression under normal circumstances[222]. Targeted deletion of these cells in rat models of hepatic IRI resulted in a reduction of hepatic HO-1 expression and worsening structural and functional injury[222, 223]. Such work implies that a basal level of HO-1 expression may be important in maintaining tissue homeostasis following ischaemic injury. Conversely, addition of HO-1 macrophages may be protective in IRI. Adoptive transfer of BMDMs that were adenovirally transduced to over express HO-1 has been shown to improve outcome in murine models of renal[67, 224] and hepatic ischaemia reperfusion injury[225]. Interestingly, the HO-1 positive macrophages in these studies demonstrated a particular capacity to "home" to the site of maximal injury. Similar observations were made by Araujo et al[171] in a model of cardiac allograft transplantation which used transgenic mice that over-express HO-1. These authors commented that the capacity for these cells to effectively target and infiltrate the site of injury might be especially beneficial. It is apparent that macrophage phenotype may have important effects within IRI and transplantation systems. Findings such as those mentioned above raise important

questions as to how HA treatment might influence macrophage behaviour should this drug prove to be beneficial in IRI.

Aims

The aims of this thesis are to investigate the capacity for HA treatment to ameliorate simulated IRI within both *in vitro* and *in vivo* settings. Throughout this work, I shall focus upon changes in the physical integrity of the endothelium, given its key role in influencing the outcome following IRI. During my in vitro experiments I shall develop and characterise a model to simulate the oxygen and carbon dioxide tensions encountered during in vivo IRI. I will investigate whether pre-treatment with HA protects cells of a murine cardiac endothelial cell line within this model, and seek to establish whether any such protective effects are due to HO-1 expression by the use of specific inhibitor substances. In my in vivo experiments I will evaluate the capacity of HA administration to induce HO-1 within murine organs, and will determine in which cells and tissues this expression is maximal. In further in vivo work, I shall assess whether HA treatment is capable of protecting renal structure and function in a murine model of warm renal ischaemia reperfusion injury. In the final phase of my experiments, I will assess the capacity for HA pre-treatment to protect renal structure in a murine model of renal isograft transplantation. A key focus in these transplant experiments will be to compare and contrast the effects of HA treatment upon donor and recipient animals. At each stage of the work I shall attempt to identify the mechanisms that might underpin any protective benefit observed. I will assess structural changes within the endothelium within these in vivo models through the use of immunohistochemical techniques and will quantify changes in the populations of a variety of cell types within pre and post-operative kidneys. I will outline future experiments that would be prescient in order to clarify which mechanisms that might be responsible for the observations that I make.

Chapter 1: In vitro up-regulation of HO-1 and modification of cell injury in a model of IRI

Aims and objectives

The aims of this phase of experimental work were to establish whether HA treatment has the capacity to induce HO-1 within a murine cardiac endothelial cell line (MCEC-1). Preliminary experiments also involved developing a methodology to quantify the numbers of Hoescht counterstained cells in 12 well plates. This would enable the analysis of subsequent in vitro experiments. In subsequent experiments an in vitro model of ischaemia reperfusion injury (IRI) was established and the effects of HA treatment upon MCEC-1 cells within this model were investigated.

The aims of this chapter are:

1. Establish the capacity of HA to upregulate HO-1 in MCEC-1 cells.
2. Validate a method for quantifying "viable" Hoescht counterstained MCEC-1 cells.
3. Develop an in vitro model to simulate during IRI
4. Study the effects of HA pre-treatment of MCEC-1 cells within this system.

Materials and methods:

Chapter 1 Cell Culture and induction of HO-1 in MCEC-1 cells

MCEC -1 cells were cultured on gelatin coated T75 flasks (1% gelatin) in full MCEC-1 media – Dulbecco's Modified eagle's Medium (DMEM) and 10% Fetal calf serum (FCS) (GIBCO BRL) with penicillin 100U/ml, streptomycin 100mg/ml L-Glutamine 2mM (GIBCO BRL) and endothelial cell growth factor (ECGF 30 μl/ml, Sigma). MCEC-1 cells were cultured at 33°C and maintained in culture by passaging when the monolayer was 70-80% confluent. The MCEC-1 cells adopted a "cobble stone" appearance when confluent (Fig 1.1). Passages 13 - 15 were used for all experiments. MCEC-1 cells were plated down in gelatin coated 6 well plates with 250,000 cells per

well. Medium was refreshed after 3 hours and the cells were kept at 37°C overnight. The following day the medium was exchanged for medium containing a range of increasing concentrations of HA solution made up in full media. 0, 1, 10, 25, 50 and 100μM HA were selected. The cell plates were shielded from natural light by tin foil due to the photo instability of HA and returned to the 37°C incubator for 6 hours. The medium was then removed, the wells were washed with PBS and the plates were stored at -20°C until required for protein analysis by western blotting.

Western Blotting of HO-1 protein

Tissue samples were prepared by homogenization in buffer (50mM Tris, 20mM NaCL, 10mM KCL, 0.1mM dithiothreitol, 1mM EDTA, 1% SDS, pH 7.4) supplemented with commercially available anti-protease tablets (Amersham, UK) and subjected to three cycles of freeze thawing. Specimens were centrifuged at 10,000g for 10 minutes. Protein concentrations were measured on the liquid phase by the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). 20 μg of protein was mixed with equal volumes of 2x laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl) prior to heating to 95°C for 5 minutes. Western Blotting was then performed in the following manner. Protein samples were loaded onto 4 to 12.5% SDS-PAGE gels, separated by electrophoresis and transferred electrophoretically onto Hybond-P membranes (Amersham, Arlington Heights, IL). The membranes were blocked with 5% dried milk in PBS for 30 minutes prior to incubation overnight at 4°C with polyclonal rabbit anti-rat HO-1 antibody (1:5000 dilution; Stressgen Biotechnologies, Vancouver, Canada). This antibody is specific for the HO-1 isoform and does not cross react with either HO-2 or HO-3. After washing in TBS+0.1%Tween the blots were incubated with secondary antibody (HRP conjugated goat anti rabbit, Sigma) for 1h at room

temperature, followed by further washes in TBS+Tween. Membranes were treated with the ECL Plus chemiluminescence system (Amersham biosciences) and exposed to blank radiographic film (Kodak). Films were developed using an Amersham Hyperprocessor automated developer. Protein loading was confirmed by reprobing the membranes with anti β-actin antibody (Sigma).

Results

Analysis by western blotting showed a dose response type relationship between the concentrations of HA administered and HO-1 induction. Maximal induction of HO-1 was achieved at 25μM HA. At higher concentrations HA appeared to be toxic to MCEC-1s. Light microscope images of the cells at these concentrations showed features of cell lysis with large clumps of cell debris and non-adherent cells visible in the wells, which implied a toxic effect. This cell death may explain the reduction in HO-1 expression observed on western blotting from cells treated with higher concentrations of HA (Fig 1.2). The MCEC-1 cell line demonstrated a degree of constitutive HO-1 expression, as evidenced by a faintly positive band at 32kDa in the lysates from cells treated with 0μM HA. Such findings might be anticipated as a consequence of the minor degrees of cellular stress inevitably encountered during culture conditions. A concentration of 10μM of HA was selected for use in future in vitro experimentation as it achieves sufficient HO-1 induction without apparent adverse toxic effects.

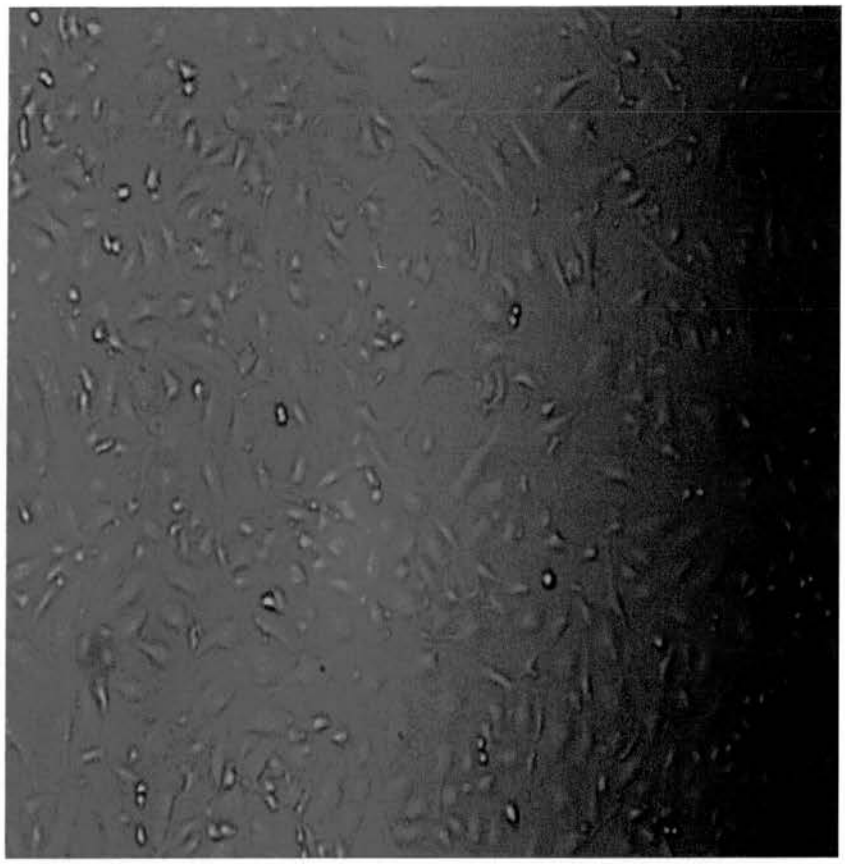


Fig 1.1. Light microscope images of MCEC-1 cells. Cells were maintained in full DMEM medium with 10%FCS. The cells adopted a "cobblestone" appearance in culture.

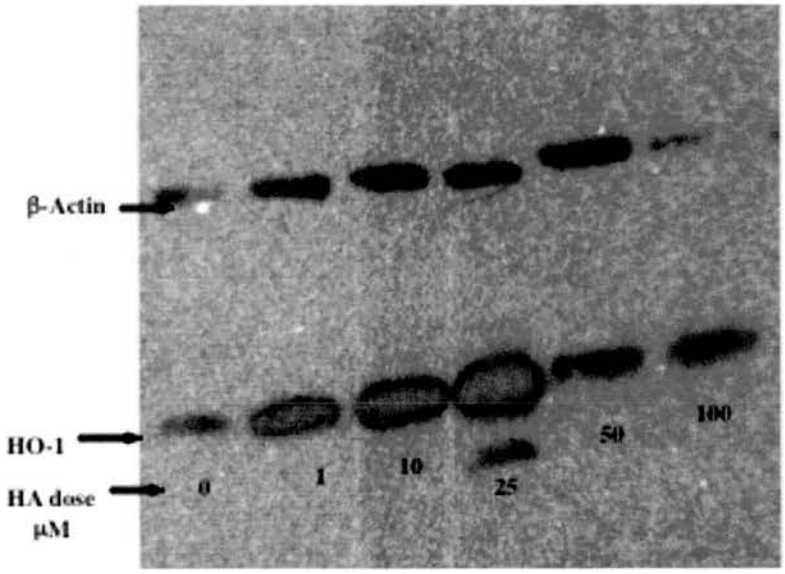


Fig 1.2 HO-1 induction in cultured MCEC-1 cells. Maximal induction is achieved at a dose of 25μM. Doses above this appeared to be toxic to the cells.

Quantification of viable MCEC-1 cells

In order to quantify the results from subsequent simulated IRI experiments, it was first necessary to develop an accurate method for counting the viable cells remaining on a culture plate following exposure to an injurious stimulus. In order to achieve this, the response of MCEC-1 cells to increasing concentrations of a known toxic agent were assessed. Puromycin was selected for this purpose. Puromycin is an aminonucleoside antibiotic utilised in cell biology as a selective agent that inhibits protein translation within prokaryotic and eukaryotic cells. Any quantification method based upon manual counting must be robust enough to accommodate a tendency for cell death to occur in an uneven distribution pattern in cell culture.

Method

MCEC-1 cells were plated onto gelatin coated 12 well plates at a concentration of 100,000 cells per well. Cells were left to adhere overnight at 33°C. The following day the medium was exchanged for standard medium containing 0, 0.25, 0.5, 0.75, 1.5 or 10 mcg/ml of Puromycin. Plates were maintained at 37°C for 24 hours and fixed with 200µl of 39% formaldehyde with methanol at 4°C for 24 hours. The wells were then washed twice with PBS and treated with 500µL of a solution containing 1 mcg/ml of Hoescht 33342 for 20 minutes. During this time plates were protected from natural light and kept at 4°C. Wells were then washed twice with PBS and coated with 75µL of a solution containing glycerol and a light quenching agent prior to image capture on an inverted microscope.

Image capture

Wells were photographed at x200 magnification on an inverted microscope. All the images were stored under coded numbers and quantification of the stored photographic images was undertaken at a later date to ensure that the analysis was performed in a blinded fashion. Wells were roughly subdivided into 10 positional "zones" (Fig 1.3). A single image was taken at random from each of these areas in each well. This technique was employed to provide a sufficient quantity of images, with an even representation of the well that would be consistent between runs of the experiment.

Results

Exposure to increasing concentrations of Puromycin resulted in a significant reduction in the numbers of "viable" MCEC-1 cells remaining within wells on completion of the experiment (Fig 1.4, Mean no. of viable cells/hpf: 20.7 ± 1.9 vs. 11.8 ± 1.6 vs. 7.3 ± 1.7 vs. 4.1 ± 0.8 vs. 0.8 ± 0.2 vs. 0.1 ± 0.06 vs. 1.4 ± 0.3; 0 vs. 0.25 vs. 0.5 vs. 0.75 vs. 1 vs. 5 vs. 10 mcg/ml puromycin; $p<0.05$, $n=3$). Moreover, the graph follows a smooth "dose response" type pattern, as might be anticipated from such an experiment. These findings suggest that this is a robust method for assessing viable MCEC-1 numbers.

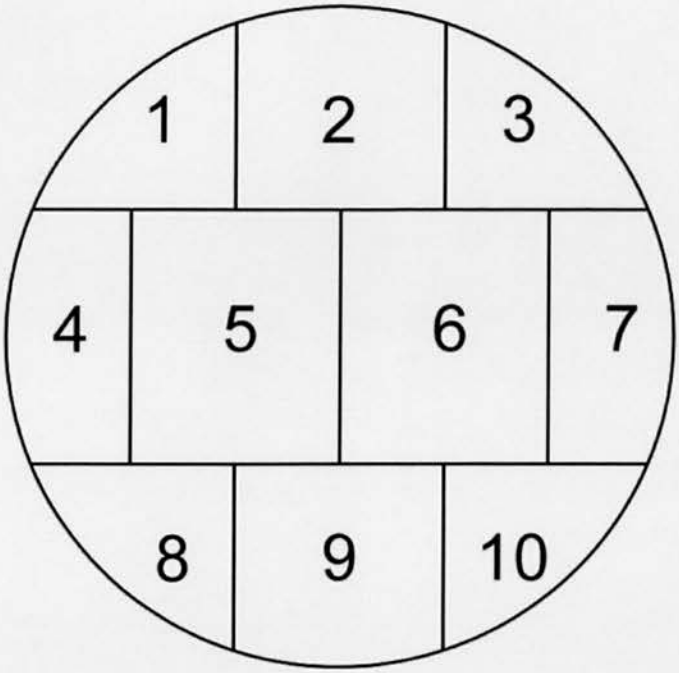


Fig. 1.3 Schematic diagram illustrating the 10 positional "zones" from which photographic images were taken for quantification.

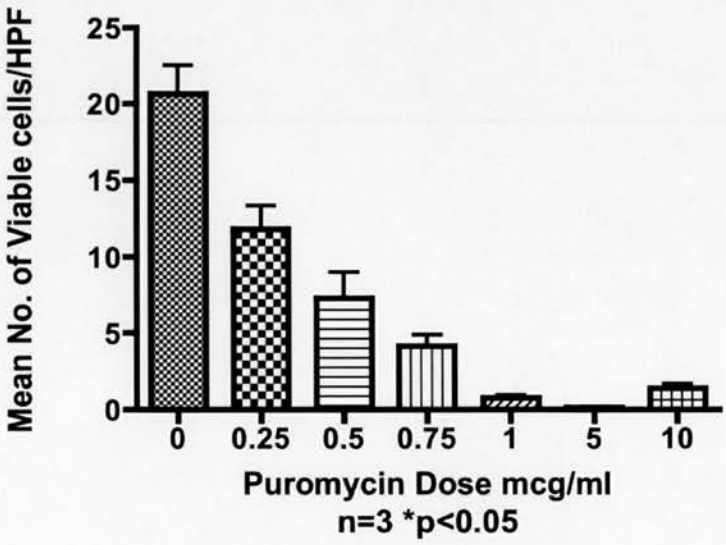


Fig 1.4. Dose response curve demonstrating a significant reduction in the numbers of viable MCEC-1 cells with increasing Puromycin dose (n=3 $p<0.05$)

In vitro model to simulate the conditions of IRI

Early work focussed on attempting to devise an in vitro experimental model to simulate the changes in gaseous tensions that are encountered within tissues during periods of ischaemia and subsequent reperfusion.

Time course exposure to simulated IRI

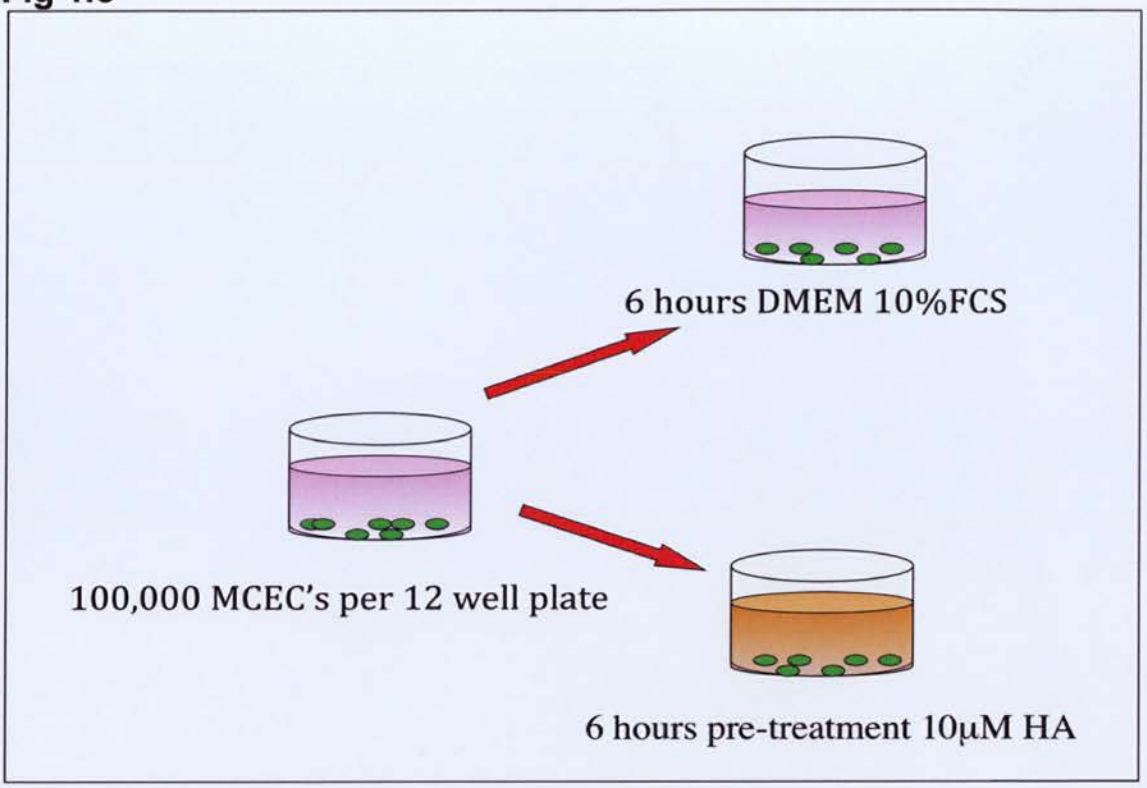
MCEC -1 cells were cultured as described previously. 100,000 cells were plated into 12 well plates that had been pre-coated with 1% gelatin and contained DMEM with 10% FCS. The cells were left to adhere overnight at 37°C. One plate was then returned to the 21% O_2, 5% CO_2 incubator as a "normoxic" control. The remaining plates were placed immediately in a Biospherix chamber that was calibrated to gaseous tensions of 0.5% O_2 and 11.5% CO_2 (Biosperix Ltd, New York, USA). These conditions are referred to as being those of "hypoxia and hypercarbia" (HCR). Upon placing the plates within the Biospherix chamber, the wells were immediately refreshed with medium that had been "degassed" for 20 minutes under conditions of HCR (0.5% O_2 and 11.5% CO_2). Experimental groups included exposure to HCR for periods of 8, 16 and 24 hours. In early experiments blood gas analysis was performed on the medium at this point to confirm the oxygen and carbon dioxide concentrations. Upon completion of the timed exposure to HCR, the medium in all the plates was replaced with 1 ml DMEM with 10% FCS that had been maintained in room air gaseous tensions. All the plates were then returned to the 21% O_2, 5% CO_2 incubator for a further 24 hours. This was intended to simulate the reperfusion phase of IRI in which exposure to increased Oxygen tensions leads to production of harmful reactive Oxygen species. At the end of the experiment the cells were fixed with formaldehyde (4% final concentration) and remaining adherent cells were stained with Hoechst 33342 (Schematic of experimental procedure in Fig 1.5). 10 high-power field photographs per well were taken as previously described under inverted fluorescent

microscopy (X 200 objective). Results were expressed as counts of viable cells per high-powered field.

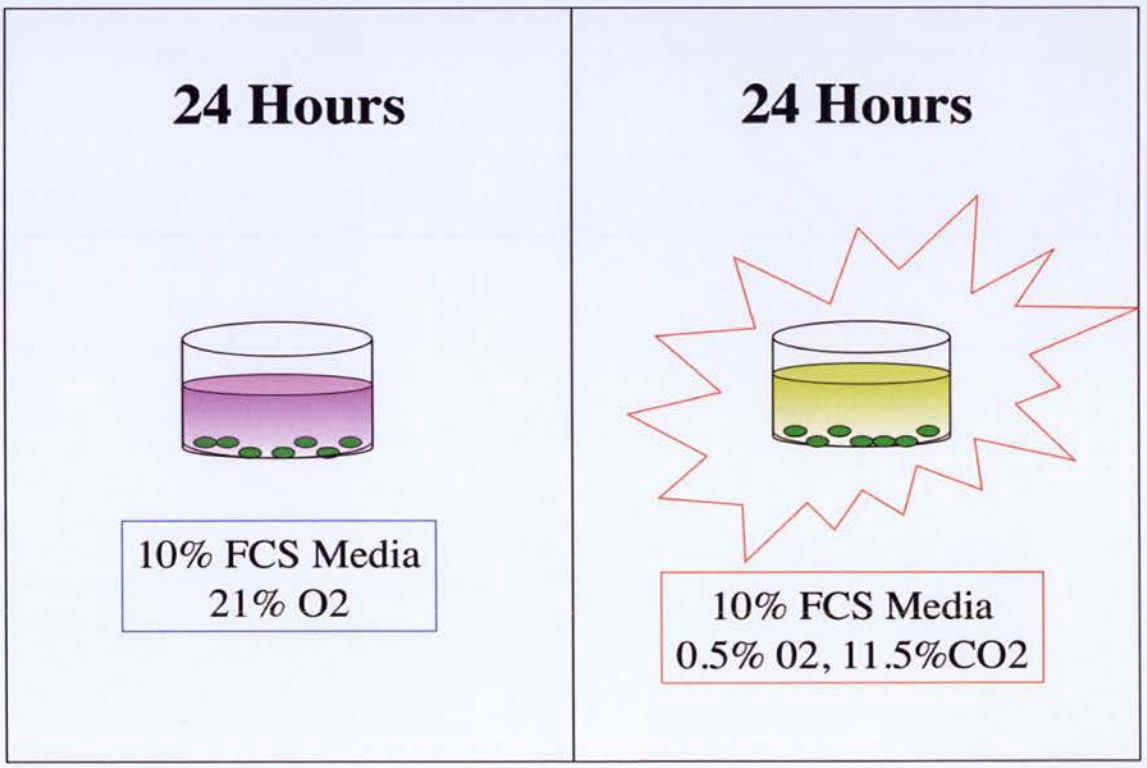
Results

This experiment confirmed that MCEC-1 cells were significantly injured when subjected to conditions of HCR as indicated by a reduction in the numbers of viable MCEC-1 cells and the presence of apoptotic bodies and cell debris in images taken from wells exposed to (HCR) (Fig 1.6). It was noted that there was a consistent tendency for the cell death to occur in an uneven fashion from the top right of the well (zone 3) progressively toward the centre (zones 5-6) and then toward the bottom left (zones 5,6,8,9). The reasons for this effect remained unexplained. This pattern confirmed the necessity of capturing images from defined areas as indicated in earlier methods. A significant reduction in the numbers of viable MCEC-1 cells per high-powered field was seen upon exposure to HCR for periods of 16 hours, compared to those cells maintained in normal oxygen tensions (31.5±1.0 vs. 17.33±2.7; Normoxia vs. 16 hours HCR; $n=3$, $p<0.05$) and 24 hours (31.5±1.0 vs. 1.6 ± 0.78; Normoxia vs. 24 hours HCR; $n=3$, $p<0.05$) but not at the 8-hour time-point (31.5 ± 1.0 vs. 25.6 ±7.3; Normoxia vs. 8 hours HCR; $n=3$, $p=0.7$) (Fig 1.7). A 24-hour time point was selected for the purposes of future experimentation using this model. This time point selected for use in future experiments as it was practical in terms of organisation of experiments and was the point at which the most significant level of injury was observed.

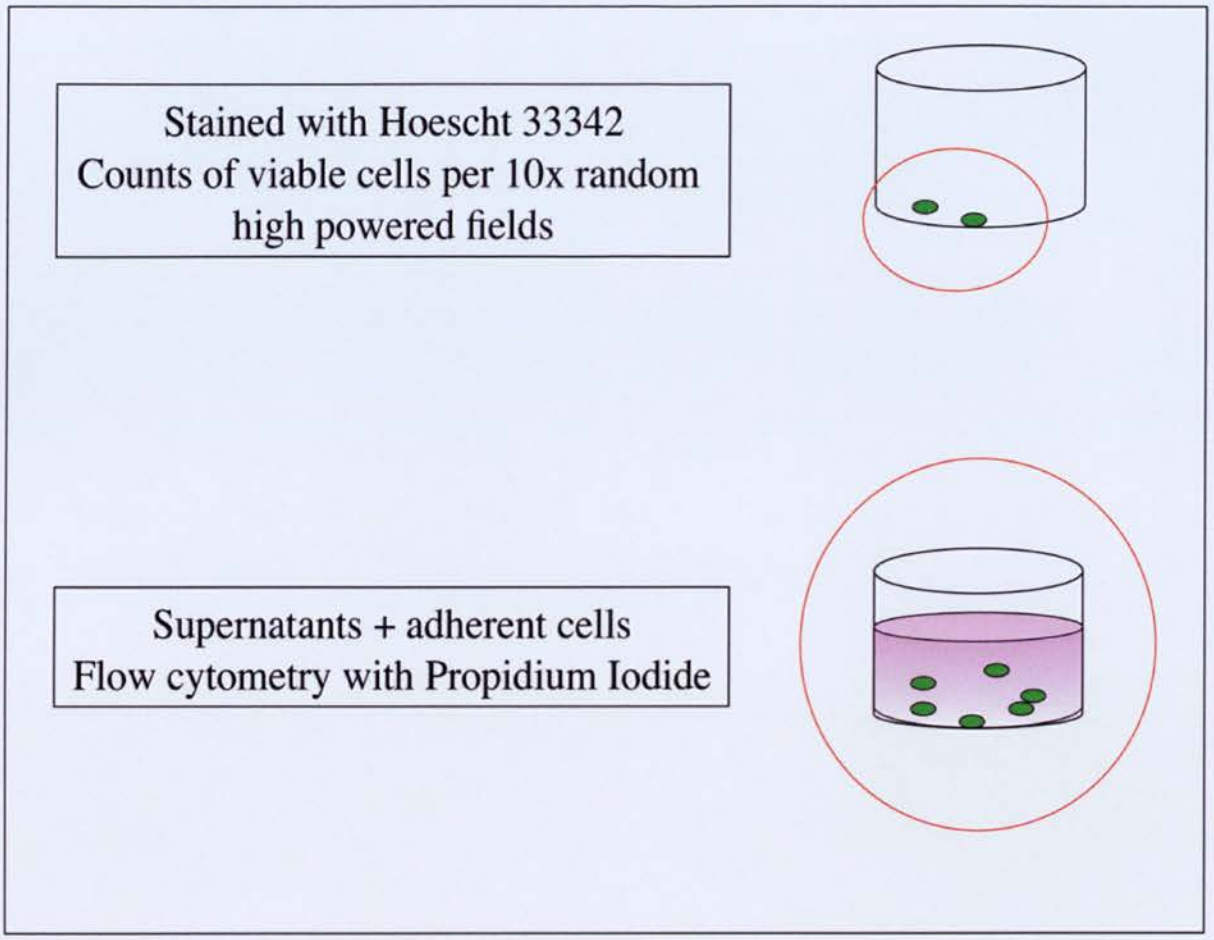
Fig 1.5



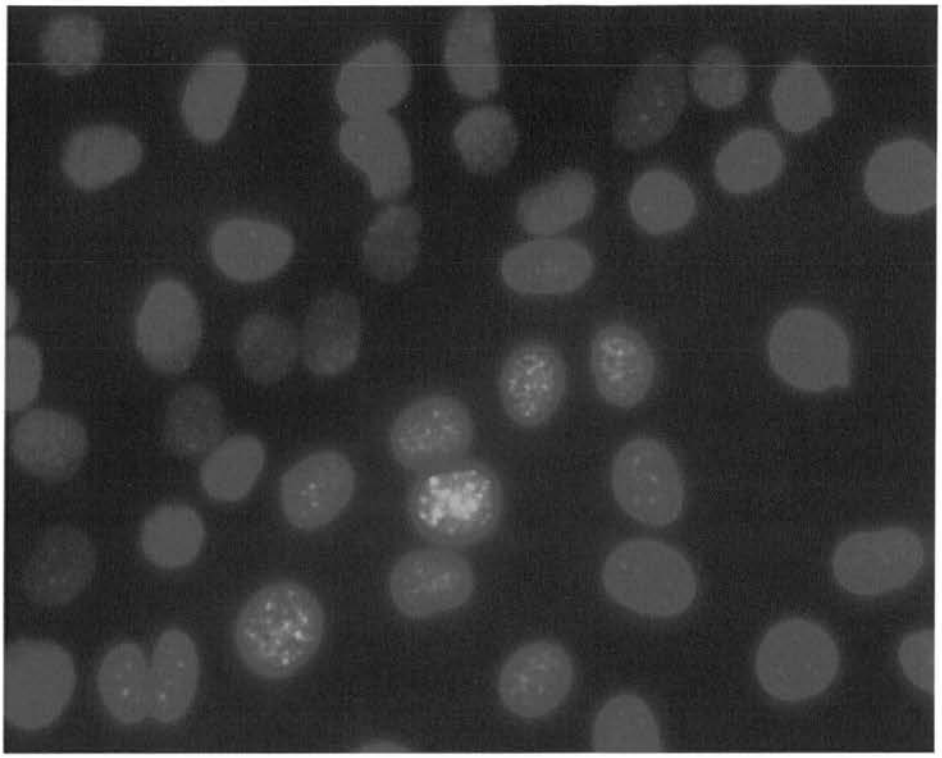
Stage 1: 100,000 MCEC-1 cells were added to 12 well plates. After being left to adhere for 6 hours, half the wells were treated with 10μM HA. Medium on the control wells was refreshed with DMEM containing 10% FCS.



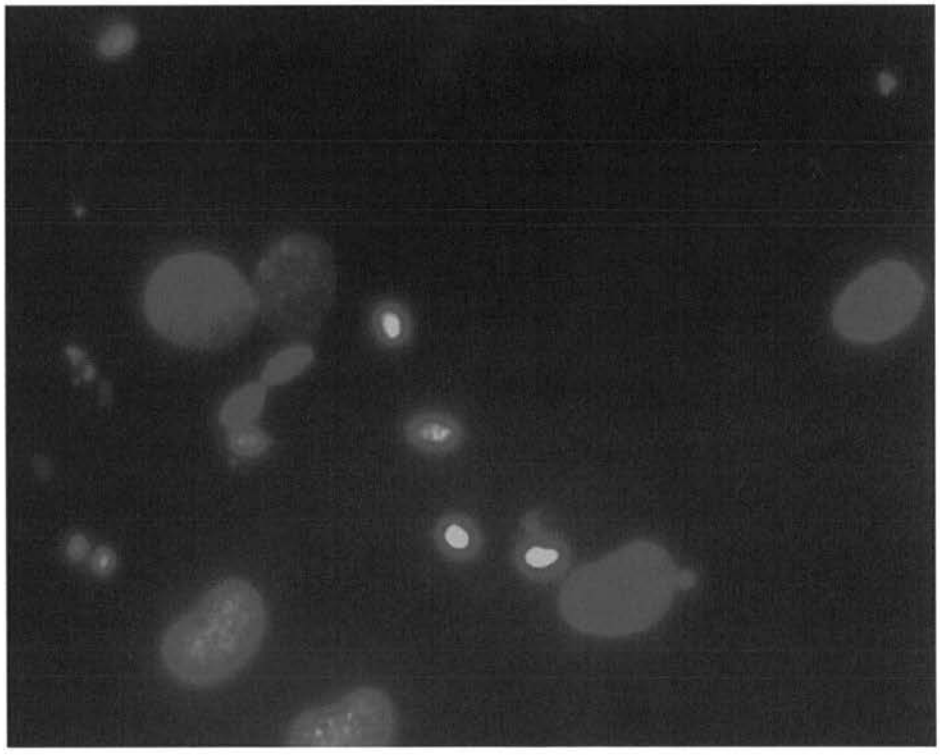
Stage 2: Plates were placed either in a hypoxic chamber equilibrated to 0.5% O2 and 11.5% CO2 (HCR) or within an incubator with containing normal Oxygen and Carbon Dioxide concentrations (Normoxia). After 24 hours, all the plates were returned to normoxic conditions and the medium in all wells was refreshed. After a further 24 hours the experiment was concluded.



Stage 3: Cells were fixed with formaldehyde (4% final concentration) at the conclusion of the experiment. Readouts of viable Hoescht stained cells adherent to the base of the well were obtained. In subsequent experiments the contents of the whole well were analysed by flow cytometry with Propidium iodide.



24 hrs Normoxia



24 hrs HCR

Fig 1.6: Cells maintained in Normoxia and stained with Hoescht compared with those exposed to 24 hours HCR. HCR exposed cells are markedly fewer in number. Cell debris and apoptotic bodies are also identified in this field.

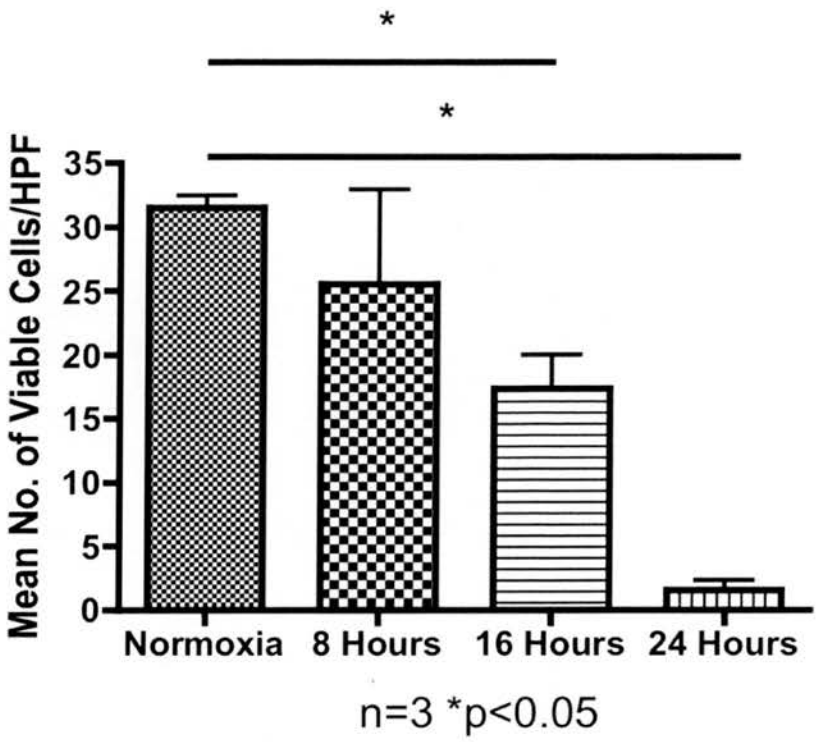


Fig 1.7: Time- course experiment demonstrating significant reductions in the number of viable MCEC-1's following exposure to 16 and 24 hours HCR (n=3, $p<0.05$)

The effect of Heme arginate pre-treatment on MCEC-1 cell number following exposure to simulated IRI

Method

MCEC-1 cells were cultured as described previously. 100,000 cells were plated into 12 well plates in DMEM with 10% FCS. The wells were pre-coated with 1% gelatin. Cells were left to adhere overnight at 37°C. Half the wells were then treated for 6 hours with 2 mls of 10μM Heme arginate (Normosang; Leiras Oy Pharmaceuticals, Finland) in DMEM with 10% FCS. Control wells received 2mls of full media. After 6 hours the medium on all the plates was refreshed with DMEM with 10% FCS. Half the plates were then returned to the 21% O_2, 5% CO_2 incubator. The other plates were refreshed with medium that had been degassed for 20 minutes in hypoxic conditions (0.5% O_2, 5% CO_2). These plates were then placed immediately in conditions of hypoxia and hypercarbia for 24 hours (0.5% O_2 11.5% CO_2, Biospherix chamber, Biosperix Ltd, New York, USA). The medium on all wells was then replaced with 1 ml DMEM with 10% FCS and all plates were returned to the 21% O_2, 5%CO_2 incubator for a further 24 hours. Cells fixed with formaldehyde at the end of the experiment (4% final concentration) and the remaining adherent cells were stained with Hoechst 33342. 10 high-power field photographs per well were taken under inverted fluorescent microscopy (X 200 objective). Results are expressed as counts of viable cells per high-powered field. In subsequent experiments, performed in a similar manner, cells remained unfixed. In these experiments the supernatants were harvested and combined with adherent cells that had been gently removed from the base of the well after trypsinisation (100μl trypsin for 10 seconds followed by blocking with DMEM with 10% FCS) and gentle agitation with a pastette. The cells were stained with propidium iodide (Sigma) and analysed by flow cytometry. Results were

expressed as % propidium iodide-positive cells. Experiments were subsequently repeated in a similar fashion with the inclusion of two control groups pre-treated with full medium containing 10μM HA and 50μM of Zinc protoporphyrin (one maintained in normoxia, the other in conditions of HCR). Upon completion of the experiment these plates were stained with Hoescht 33342 and then photographed under the inverted microscope and counted as described previously.

Results

Counts of viable cells per random high-powered field were reduced in untreated cells following exposure to HCR when compared with untreated cells maintained at normal oxygen tensions. Cells pre-treated with 10μM HA were significantly protected against conditions of HCR (Fig 1.8; 30.9 ±1.7 vs. 7.0 ± 3.2 vs. 20.0 ±3.1; Normoxia vs. HCR vs. HA+HCR; n=4 $p<0.05$). In subsequent experiments, a reduction in positive PI staining was observed following treatment with HA prior to HCR when compared with controls (Fig 1.9; % positive PI staining; 6.8% vs. 78.7% vs. 43.4%; Normoxia vs. HCR vs. HCR+HA; representative figures from one experiment). In later experiments that included ZNPPiX treatment groups, the protective effect of HA pre-treatment was replicated (Fig 1.10; 32.9 ± 0.7 vs. 1.5 ± 0.7 vs. 20.7 ± 0.5 Normoxia vs. HCR vs. HA+HCR; n=3, $p<0.05$). A reduction in cell number was also seen in cells that were maintained at normal oxygen tensions and received HA and ZNPPiX when compared to cells that received HA alone (Fig 1.10, 32.6 ± 0.5 vs. 22.4 ± 2.8; Normoxia + HA vs. Normoxia+HA+ZN; n=3, $p<0.05$ unpaired t-test). There was also a clear trend toward abrogation by ZNPPiX of the protective effect of HA in this experiment although this did not reach statistical significance (17.8 ± 3.2 vs. 8.1 ± 2.4; HCR+HA vs. HCR vs. HA+ZN; n=3 p=0.07).

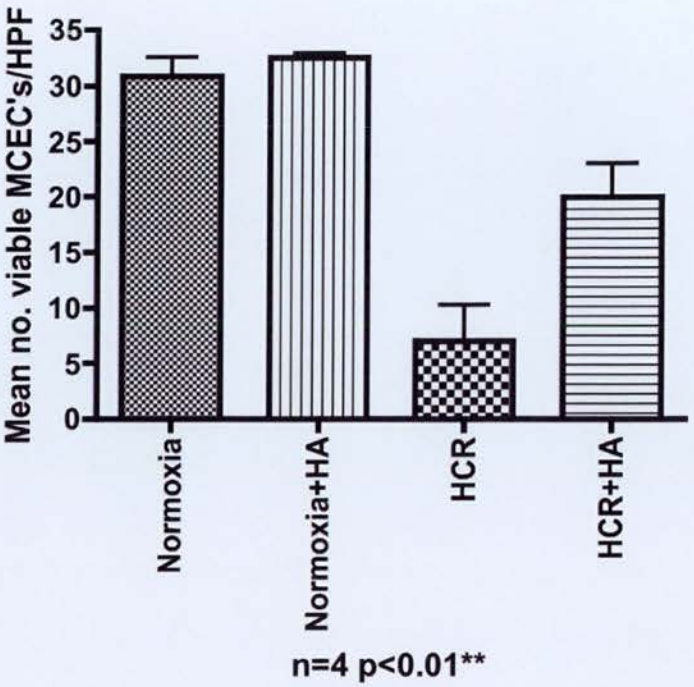


Fig 1.8. Significantly fewer viable MCEC-1 cells remain after 24 hours exposure to HCR compared with normoxic controls. Cells pre-treated with 10μM HA are significantly protected from this injury (n=4, $p<0.05$)

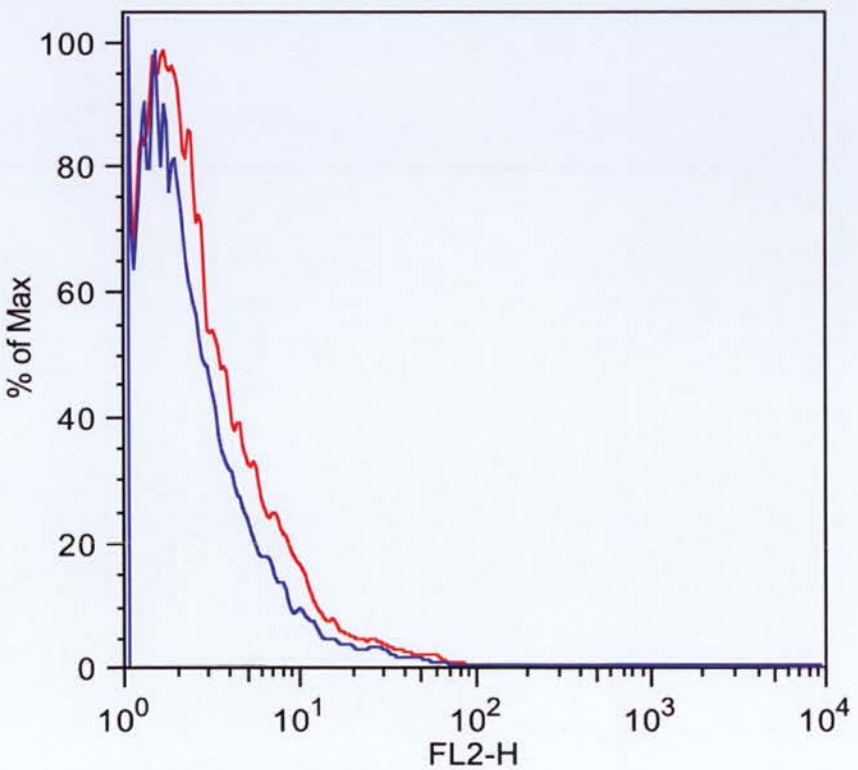


Fig 1.9 - Normoxia. Cells maintained at normal Oxygen tensions for 24 hours exhibit similar degrees of positivity for PI. Red line - untreated cells, 6.82% PI positive: Blue line - cells pre-treated with 10μM HA for 6 hours, 4.93% PI positive. Representative plot taken from 3 experiments.

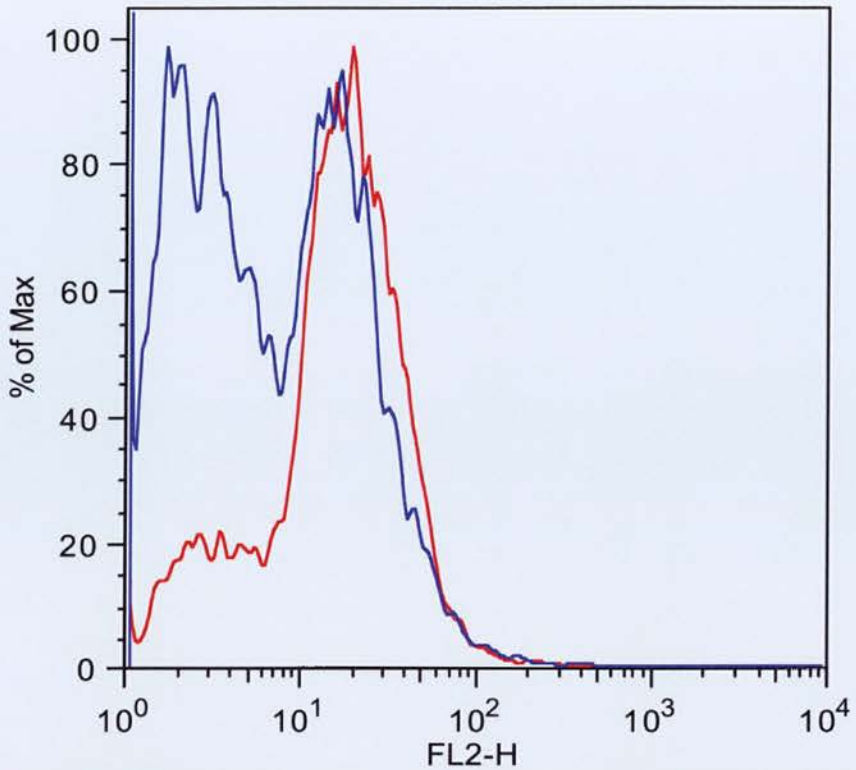


Fig 1.9 - HCR. Cells exposed to conditions of HCR for 24 hours. Red line - untreated cells, 78.7% PI positive: Blue line - cells pre-treated with 10μM HA for 6 hours, 43.4% PI positive. Representative plot taken from 3 experiments.

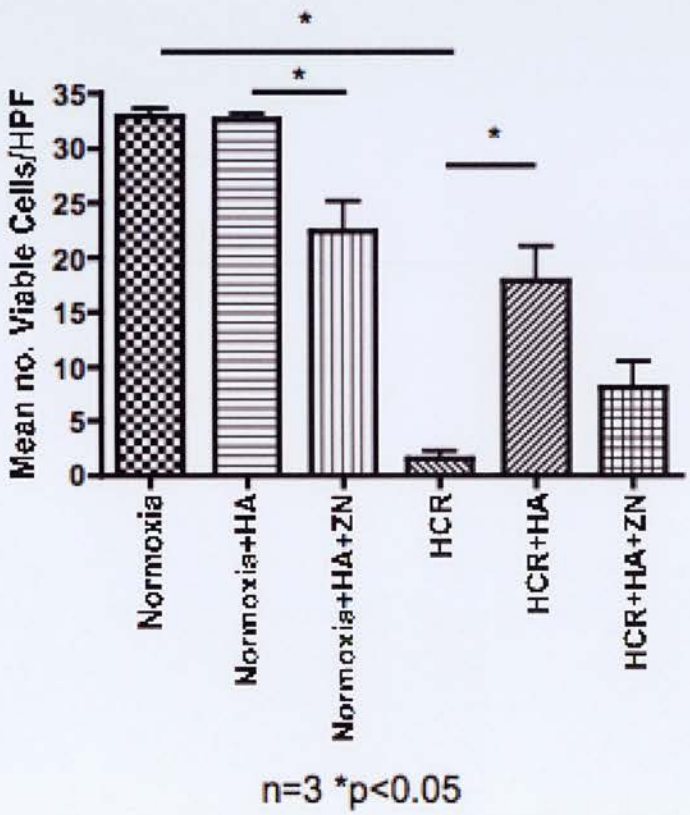


Fig 1.10 – Pre-treatment with 10μM of HA significantly protects MCEC-1 cells exposed to 24 hours HCR (n=3, $p<0.05$). Cells maintained in normoxia and treated with HA and Zinc were significantly fewer when compared to those cells that just received HA treatment (n=3, $p<0.05$). There was a trend toward an abrogation of the protective effects of HA in cells that received ZNPPiX, a specific inhibitor of HO-1 (n=3, $p=0.07$)

Summary

These data demonstrate that:

1. HA treatment of MCEC-1 cells induces HO-1.
2. MCEC-1 cells that received pre-treatment with HA were more numerous and exhibited less cell injury (as measured by PI staining) following simulated IRI.
3. Use of a specific inhibitor of HO-1 appeared to abrogate this protection. Although the differences between the groups fell short of statistical significance, it does seem to imply that HO-1 activity may be responsible for the protective effects observed.

Discussion

Primary endothelial cell lines have traditionally proved difficult to isolate, the techniques required to do so being both time consuming and costly. Such cultures often yield only small numbers of cells that do not readily withstand the processes of passaging, frequently becoming senescent at low passage numbers. This has limited their use in a variety of experimental conditions. The use of immortalized cell lines is associated with undesirable phenotypic changes such as reduced responsiveness to cytokine stimulation[226] and the loss of surface antigens[227]. Furthermore, immortalized cell lines are unsuitable for the study of endothelial cell proliferation. In these experiments I used the murine cardiac endothelial cell line (MCEC-1) that was originally described by Lidington et al[228]. These authors successfully isolated growth factor responsive cardiac endothelial cells from a strain of transgenic mice (H-2Kb-tsA58[228]). MCEC-1s are characterised by their capacity to form tubules and by their expression of CD31, ICAM-2 and endoglin. MCEC-1s also harbour a temperature sensitive simian virus 40 large Tag gene under H-2K class 1 promotor control, which ensures that they proliferate rapidly when incubated in permissive conditions (33°C with IFNγ). Proliferation was reduced by 30-fold when the cells were exposed to non-

permissive conditions (38° without IFNγ). This temperature sensitivity makes the proliferation of the cells easy to regulate under laboratory conditions and enhances the practicality of the cell line. Lidington et al also demonstrated a capacity for these cells to express E- and P-selectin, ICAM-1 and VCAM-1 in response to stimulation with the cytokines TNF α and IL-1β. This contrasts with the author's previous experience with immortalized human endothelial cell lines such as HMEC-1, ECV304 and EaHy926 which failed to show such a response to cytokine stimulation[226]. These characteristics make the MCEC-1 cell line a practical tool to study endothelial cell function in response to inflammatory stimuli. Indeed the MCEC-1s have been used to demonstrate that TIMP-3 regulates the release of the soluble ectodomain of VCAM-1 from cytokine stimulated endothelial cells[229]. Whilst MCEC-1 cells have been shown to express CD31 by the original authors, this positivity appeared to be lost with increasing passage numbers. This has been suggested to be due to enzymatic digestion by trypsin[230]. This loss of CD31 expression has raised the question as to whether these cells can truly be regarded as endothelial cells or whether they de-differentiate from their original phenotype as they are passaged. Accordingly, only low and similar passage numbers were used in this series of experiments. The extent to which the biology of MCEC-1s may mimic responses in renal endothelial cells is also an important question. Endothelial cells derived from different anatomical locations may display differing structural and functional characteristics (reviewed in[231]). Craig et al found intrinsic structural differences between endothelial cells derived from capillaries compared with those from large vessels[232]. In this paper, only endothelial cells derived from brain capillaries were shown to possess tight junctions by electron microscopy, whereas only the endothelial cells derived from large vessels (in this case the aorta) contained Weibel-Palade bodies. Gumkowski et al[233] demonstrated significant functional heterogeneity between microvascular endothelial cell cultures

derived from a variety of murine organs including lung, liver, brain, heart, placenta and kidney. These cell lines differed in their expression of cell surface antigens, angiotensin-converting enzyme and by differential binding of various plant lectins. Such differences are likely to reflect the range of functions performed by the endothelium at each site and are perhaps to be expected. This heterogeneity highlights the complexity of the vascular bed as a whole and has implications for how relevant data obtained from a cell line from one organ may be to the behaviour of endothelial cells within another organ system. There are of course inherent difficulties in extrapolating findings made in vitro to those that may be made in vivo in any case. Unfortunately there remains a paucity of commercially available renal endothelial cell lines at the present time. The notable exception to this is the REC-A4 line that is derived from renal microvascular endothelium obtained from normal mouse tissues[234]. These cells retain morphological and ultrastructural similarities with resting microvascular endothelial cells. However, the use of REC-A4 cells is currently not widespread outwith the institution that initially characterised the cell line.

Attempting to accurately model the phenomenon of ischaemia reperfusion injury in vitro is immensely challenging. IRI is a complex in vivo process that consists of an ischaemic phase of variable duration, followed by a reperfusion phase that is associated with the generation of reactive oxygen species and a subsequent cellular influx. This infiltrate is composed of differing populations of cells that are recruited in a specific temporal sequence. The present model differs from in vivo IRI several respects. The real physical structure of the microvasculature is composed of endothelial cells that form 3 dimensional, branching tubular structures, not a single cell monolayer as is represented in these experiments. The model does not reproduce the temperature variations to which organs are exposed prior to transplantation. The model also does not replicate the effects of turbulent blood flow that are encountered

in vivo, which may be relevant as sheer stress has been reported to play a role in HO-1 induction in the endothelium[235]. In reality transplanted organs are unlikely to be exposed to such lengthy periods of ischaemia. The 24-hour exposure time point used in these experiments was selected upon the basis of the level of injury that results, and the practicality of that time-point for experimental purposes. More limited ischaemic times may result in a lesser degree of endothelial cell loss and greater degrees of endothelial cell activation and endothelial dysfunction[191, 194]. This may have some bearing upon the nature of the subsequent injury, with effects such as the loss of barrier function, cellular infiltration, vasoconstriction and thrombosis potentially playing more important roles than cell loss through necrosis per se. The changes in oxygen and carbon dioxide concentrations that are used in this model replicate only one component of the real injury; accordingly, this model does not reflect the entirety of the processes that occur during IRI in vivo. Such events are almost impossible to model accurately within the laboratory setting, the number of variables required to do so would result in highly complex, impractical experiments. The majority of studies attempting to model IRI in vitro focus upon one component of the injury. Harrison et al[236] demonstrated that heat shock protein 90 binding agents protect a human renal adenocarcinoma cell line (ACHN) against the oxidative stress induced by exposure to Hydrogen peroxide (H_2O_2) mimicking the generation of free radicals encountered when the oxygen rich blood supply is restored during the reperfusion phase of IRI. Others have manipulated gaseous tensions to simulate the processes of ischaemia followed by reperfusion, as I have done. Zenebe et al[237] using an air-sealed chamber infused with either air or Nitrogen, demonstrated that hypoxia followed by re-oxygenation caused cytochrome c release and oxidative stress in cardiac mitochondria via a nitric oxide synthase dependent mechanism. Other researchers have replicated the low temperatures to which organs are exposed during periods of storage prior to implantation[238].

One of my central hypotheses is that endothelial cell damage forms a critical early event in IRI. Loss of endothelial cells and physical disruption of the microvasculature are important factors that predispose organs to ongoing downstream ischaemic damage[66]. This model, albeit crude, does reproduce the ischaemic conditions encountered during in vivo IRI and allows us to evaluate how those conditions affect endothelial cell survival. It also allows us to assess how HA modulates this injury. The settings for the Biospherix chamber used for these experiments were selected in accordance with a clinical study that measured the partial pressures of Oxygen and Carbon Dioxide within clamped ischaemic organs during surgical procedures[239].

The simulated IRI experiment outlined in this chapter was analysed using two different readouts. The use of viable cell number counts and Propidium iodide (PI) provide measures of both cell survival and cell injury respectively. Propidium Iodide is an intercalating agent that binds to double-stranded DNA, but can only cross the plasma membrane of non-viable cells. As such, cells that are positive for PI have compromised membrane integrity. Further sub classification into cells that are undergoing necrosis and apoptosis is possible by alteration of the settings upon the flow cytometer, or by the additional use of Annexin V, which binds to the phosphatidylserine that becomes exposed on the surface of cells undergoing apoptosis. The results presented here represent merely PI positive cells and as such include both apoptotic and necrotic cells. It may be of interest to attempt to differentiate between these modes of cell death given the emerging evidence attesting to the immunomodulatory effects of apoptotic cells[213]. It should be noted that each read-out also analyses a slightly different aspect of the experiment. Only those cells that remain adherent to the base of each well are visualized and included in the "viable cell counts", the supernatents that may contain injured endothelial cells that might be

dislodged from the monolayer were disregarded. In contrast, the entire contents of the well were analysed by PI staining in subsequent runs of the experiment. It seems likely that the contents of the supernatent will largely consist of debris and injured cells that have become detached from the monolayer. It is therefore doubtful that the contents of the supernatent will alter counts of viable cells as such. Enzymatic digestion and well scraping both have the potential to injure cell membranes and therefore increase positive PI staining, giving an erroneous impression of increased injury levels. Accordingly, great care was taken when preparing cells for flow cytometric analysis to ensure that all groups were exposed to short, identical periods of trypsinisation prior to quenching with medium containing 10% FCS. The use of two separate readout measures in this system strengthens the evidence for a protective effect of HA. HA pre-treatment was shown not only to reduce cell injury, but also to increase the number of remaining viable cells.

In these experiments, Zinc Protoporphyrin (ZZPPiX) was used as a specific inhibitor of HO-1. This agent is widely employed in the literature on HO-1 in this capacity[90, 93, 173]. The use of metalloporphyrins in the investigation of a physiological role for HO-1 has been criticized due to their capacity to inhibit NOS and sGC activity[240-242]. Appleton et al[243] have shown, however, that ZNPPiX when administered at a concentration of 50 μM can inhibit HO activity without significantly inhibiting the activity of either nitric oxide synthase (NOS) or soluble guanylate cyclase (sGC) and this dose has been commonly employed in the literature[244-246]. Within these experiments there was a significant reduction in the numbers of viable MCEC-1 cells within control groups that were maintained in normal oxygen tensions and received both HA and ZNPPiX. It is possible that this may represent a toxicity effect of ZNPPiX and therefore that the apparent trend toward abrogation of the HA mediated protection against conditions of HCR might be due to toxicity of the inhibitor rather

than inhibition of the protective effects of HO-1. An alternative explanation might be that a level of constitutive expression of HO-1 is required to protect these cells against a variety of stresses that they encounter routinely during cell culture, or to prevent a degree of apoptosis that these cells might otherwise normally undergo[174]. Reference to Fig 1.1 indicates that untreated MCEC-1 cells do express HO-1 when they are maintained in 12 well plates. Such expression may be necessary for their survival. Future work to establish the effects of ZNPPiX might include HO-1 activity assays to verify functional HO-1 induction by HA and functional inhibition by ZNPPiX administration. Alternatively, short interfering RNA (SiRNA) may be utilised to specifically "knock out" HO-1 and therefore confirm that the protection seen in this system relates to an effect of HO-1 activity.

Future work

These data as presented do not explain the mechanisms responsible for the protective effect of HA administration within this experimental system. Future experimental work would principally be directed toward definitively clarifying whether enhanced HO-1 expression was implicated by the use of siRNA to "knock out" the HO-1 gene within this cell line prior to HCR. Thereafter it may also be of value to determine whether one of the individual breakdown products of HO-1 activity provides endothelial cell protection against HCR in isolation, or whether such protection is multifactorial. Several previous studies have suggested that one or more of these substances may be protective under a variety of experimental conditions[247-250] Carbon monoxide releasing molecules (CORMs) are commercially available, as are both biliverdin and bilirubin. These substances could be added individually to MCEC-1 mono-cultures immediately prior to HCR to assess their potential protective effects. Exogenous treatment with iron is unlikely to be rewarding as its therapeutic benefits may be mediated through its rapid depletion by the post-transcriptional up-regulation

of ferritin. Ferritin sequesters other Fe2+ ions in the milieu, preventing the generation of harmful reactive oxygen species (ROS) via the Fenton reaction[165, 249].

IRI is a highly complex, multifactorial phenomenon *In Vivo*. However, the basic experimental model described might be further adapted to evaluate other key aspects of the process. For example, some authors have shown that HO-1 positive macrophages may mediate the therapeutic benefits of HA treatment within an *in vivo* model of IRI in aged mice[50]. HA pre-treated mice in this study showed reduced levels of acute tubular necrosis (ATN) following simulated IRI. It would be interesting to co-culture HA pre-treated macrophages with either MCEC-1 cells or primary cultures of renal tubular epithelial cells and expose them to conditions of HCR. HO-1 over-expression induces an anti-inflammatory phenotype within macrophages[67, 251, 252] and a growing body of evidence suggests that HO-1 positive macrophages may be protective within in vivo models of IRI[50, 222, 224]. Direct and indirect in vitro co-cultures may clarify whether any therapeutic benefits occur as a result of a substance produced by HO-1 positive macrophages (such as CO) or due to direct cell-to-cell contact. Such experiments would have to be appropriately designed to address a number of potential confounding factors. Firstly, an observation from my own preliminary work was that MCEC-1 cells when plated at higher numbers and increasing levels of confluence, were more resistant to injury due to HCR. This finding may make comparison of co-culture groups with control "MCEC-1 only" groups problematic. The presence of an additional population of cells in the well at varying ratios to the MCEC-1 cells may physically shield the MCEC-1 monolayer from exposure to the conditions of HCR. My experience with this model suggested that MCEC-1 cells were more resistant to injury unless they were abruptly exposed to the degassed HCR medium. Potentially, a more gradual exposure to conditions of HCR, might occur should MCEC-1s be physically "shielded" by another cell layer

layintop of them in co-culture. This might provide the endothelial cells with sufficient time to upregulate their own cellular defence mechanisms such as HO-1. Co-cultures may therefore potentially demonstrate protection through a partly physical effect. Secondly, it is known that hypoxia results in both vascular endothelial cell growth factor (VEGF) and hypoxia inducible factor (HIF-1α) production by macrophages[253]. VEGF production might confound observations by increasing MCEC-1 numbers through proliferation. HO-1 expression has also been shown to be HIF-1α dependant[254], which is likely to result in increased HO-1 production within control groups. I undertook some preliminary direct co-culture experiments with HA treated and untreated macrophages and MCEC-1 cells under conditions of normoxia and HCR. In this work the cells were cultured at a ratio of 2 macrophages to 1 MCEC-1 cell. I attempted to analyse the results using the image capture and cell counting technique described in this chapter, the final readout being mean number of viable MCEC-1 cells per high-powered field. Staining with Hoescht alone would not distinguish between the two cell types, as all cells stained with this marker appear fluorescent under the inverted microscope. Although macrophages and MCEC-1 cells differ morphologically (MCEC-1 cells are physically larger) a more reliable way of differentiating between the two cell types was needed. I attempted to label the macrophages using "cell tracker" dyes, staining the endothelial cells orange and the MCEC-1 cells green dyes in accordance with a method originally described by Duffield et al[255]. Unfortunately I found the dyes to be a temperamental and either one or the other cell line would consistently fail to stain strongly. Even when the cells were labelled correctly it could be difficult to count viable MCEC-1 cells. The crisp, intense staining of the cells nucleus with Hoescht makes asesment of the viability of a cell relatively straightforward. In contrast, the cell tracker dyes provide less distict staining and less nuclear definition which makes counting and assessing viable cells

more problematic. In addition, the presence of macrophages within the wells partially obscured the MCEC-1 monolayer, hampering accurate quanification. I did attempt to use Hoescht staining alone for quantification in some runs of these experiments, as I thought distinction between the two cell types on size grounds alone may have been possible. The results I obtained with this method seemed to suggest that the presence of either treated or untreated macrophages resulted in a trend toward MCEC-1 preservation. However, it is clearly possible that inadvertent counting of some macrophages as viable MCEC-1 cells may explain these results. Repition of this experiment using flow cytometry to sort the cells into macrophages and endothelial cells groups and assessing PI and annexin V positivity may negate these issues and provide an accurate assessment of necrotic and apoptotic MCEC-1 cell numbers.

Endothelial cells express ICAM-1 and P-selectin upon exposure to conditions of hypoxia and re-oxygenation in vitro through upregulation of MAP kinase signalling[256-258]. Increased adhesion molecule expression has also been demonstrated in the microvasculature of human kidney allografts[46]. This endothelial activation represents a critical event in IRI and facilitates the infiltration of an injurious population of cells including neurophils[258]. The extent to which E-selectin may also play a role in renal IRI has been debated[259, 260]. Bilirubin and the depletion of free Iron associated with HO-1 activity have been shown to inhibit the ability of pro-inflammatory cytokines such as TNFα and IL-1β, to induce the expression of the adhesion molecules E-selectin, P-Selectin, ICAM-1 and VCAM-1 via inhibition of NF kappa B[261], and carbon monoxide is known to attenuate ICAM –1 expression by inhibiting NFKB[262]. Therefore, in addition to protecting the physical integrity of the endothelium, HO-1 may reduce endothelial activation and leukocyte induced injury as a consequence. It would be valuable to assess this in vitro, perhaps by using

abbreviated exposure to HCR and evaluating the expression of adhesion molecules in control groups and groups pre-treated with HA.

It might be suggested that the increased cell numbers seen in the HA treated groups in this experiment are as a result of increased proliferation, rather than protection of existing cells from the conditions of HCR. The literature is divided as to whether HO-1 expression or the breakdown products of heme metabolism can stimulate proliferation. Otterbein et al[263] showed that CO increased hepatocyte growth factor expression in hepatic stellate cells, but not in hepatocytes. Increased hepatocyte proliferation was observed when these two cell types were co-cultured together. However, CO has also been shown to exhibit anti-proliferative effects in vascular smooth muscle cells via mechanisms dependent on the activation of sGC and p38 MAPK [264, 265]. It is possible that the specific effects of this molecule may vary between organ systems and cell types. Indeed, Li Volti et al[266] suggest that HO-1 regulates the cell cycle in a cell-specific manner. Their data demonstrate that HO-1 increases endothelial cell cycling but decreases smooth muscle cell cycle progression in vitro. Formal assessment of proliferative activity, for example, by means cell cycle analysis following PI staining on flow cytometry would clarify this issue within this experimental system.

This phase of experimental work demonstrates that HA treatment upregulates HO-1 in MCEC-1 cells and protects them from conditions of hypoxia and hypercarbia similar to those encountered in IRI in vivo. Use of the specific inhibitor ZNPPiX suggests that this protection may be wholly or in part due to an effect of HO-1 activity.

Chapter 2: In vivo induction of HO-1 following HA administration

Aims and objectives

Results from the previous chapter established that HA treatment induces HO-1 expression within cultured murine cardiac endothelial cells and protects them against simulated IRI in vitro. The next phase of experimental work investigates the effect of intravenous HA injection upon HO-1 expression within mice.

The aims of this chapter are:

1. Establish the capacity for HA administration to upregulate HO-1 in vivo
2. Determine the optimum dose for use in future in vivo experiments
3. Clarify whether HA treatment can induce functionally active HO-1
4. Localise HO-1 upregulation to specific cell types within a range of organs

Determining the effect of IV Heme arginate upon HO-1 expression *in vivo*

Methods

6 week old FVB mice (Harlan, UK) were administered either 200μL of PBS or the same volume of a solution containing 3, 15 or 30 mg/kg Heme arginate solution via tail vein injection. 3 mice were tested per group. After 24 hours the mice were terminated via cervical dislocation in accordance with Home Office guidelines. Spleen, liver and kidney were retained for further evaluation. HO-1 expression was localised by immunohistochemistry. Baseline expression of the enzyme was quantified by western blot analysis. Bioactivity assays were performed to confirm functional upregulation of the enzyme.

Immunohistochemistry

Whole kidneys were cut longitudinally and either snap frozen in liquid nitrogen (for future use in bioactivity assay) or fixed in methyl Carnoy's solution (60% methanol, 30% chloroform and 10% acetic acid). HO-1 expression was assessed following antigen retrieval in citrate buffer (10mM sodium citrate, 0.05% Tween. Microwaved at 800W for 3 x 5 min). Sections were then incubated with rabbit anti mouse polyclonal antibody (1/500 dilution; 4°C overnight; Stressgen Biotechnologies, Vancouver, Canada). Primary antibody was incubated at 4°C overnight with subsequent incubation with mouse-adsorbed biotinylated rabbit anti-rat IgG (1/300 dilution; Vector Laboratories, Peterborough, UK) at room temperature (RT) for 30 minutes. After washing, sections were incubated with Vectastain ABC Elite reagent (Vector Laboratories, Peterborough, UK) for 30 minutes at RT, prior to further washing and staining with diaminobenzidine (DAB) (Dako UK, Cambridgeshire, UK). Counterstaining was performed with haematoxylin prior to dehydration and mounting. Whole mouse serum was used instead of primary antibody in sections included as negative controls.

HO-1 bioactivity assay

HO-1 bioactivity assays were performed to confirm the presence of a functionally active enzyme. Tissue samples were prepared and protein concentrations assayed as described in chapter 1. As a source of biliverdin reductase, human liver cytosol was obtained by homogenising samples of liver in buffer H (0.1M sodium citrate, 10% glycerol, pH 5), and collecting the supernatant following centrifugation at 105,000g for 30 minutes. A 400μL reaction mixture was then created, containing: 500μg of tissue protein; 1.5mg liver cytosol; 0.8mM NADPH; 2mM glucose; 6 mM phosphate; 0.2 units glucose 6 phosphate dehydrogenase; and 20μg hemin. The volume was

made up to 400μL in buffer Hs. The mixture was then incubated in the dark at 37°C for one hour. The reaction was terminated by adding 400μL of chloroform and vortexing for 20 seconds, followed by centrifugation at 10,000g for one minute. The chloroform phase was then collected, and the bilirubin content measured by spectrophotometry, using the difference in absorption at 464 and 530nm, and an absorption coefficient of $40mM^{-1}cm^{-1}$. Results were expressed as pg of bilirubin formed per mg protein per hour.

Dual immunofluorescence CD68 and HO-1

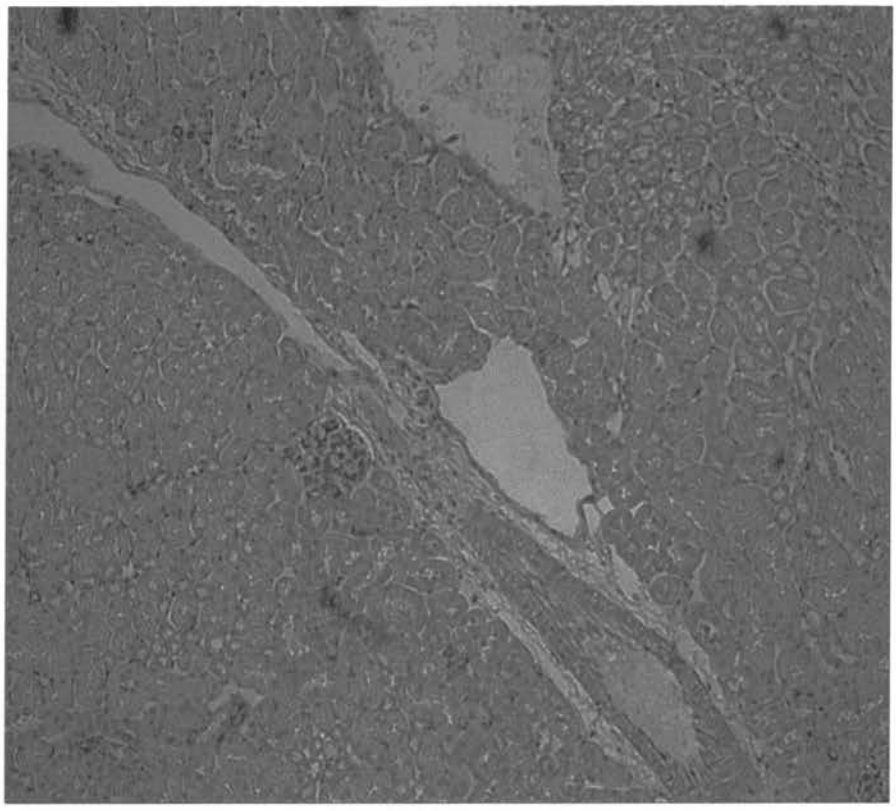
Frozen tissue was embedded in OCT mounting media and 5 micron sections cut, air dried and fixed in ice-cold acetone. After protein blockade for 30 minutes (Spring Bioscience, CA) primary antibodies (polyclonal Rabbit anti HO-1, 1:300 dilution, rat anti-mouse CD68, 1:100 dilution) were added simultaneously and incubated for 1h at room temperature. Following washing in PBS, secondary antibodies (Alexa-488 conjugated Goat anti-Rat Ig 1:250 and Alexa-594 Donkey anti-rabbit Ig 1:500, both Invitrogen) were added simultaneously for 1 hour. Slides were coverslipped with Vectashield containing DAPI (Vector Labs) prior to microscopy and image acquisition. Images analysis was performed using Photoshop CS3.

Results

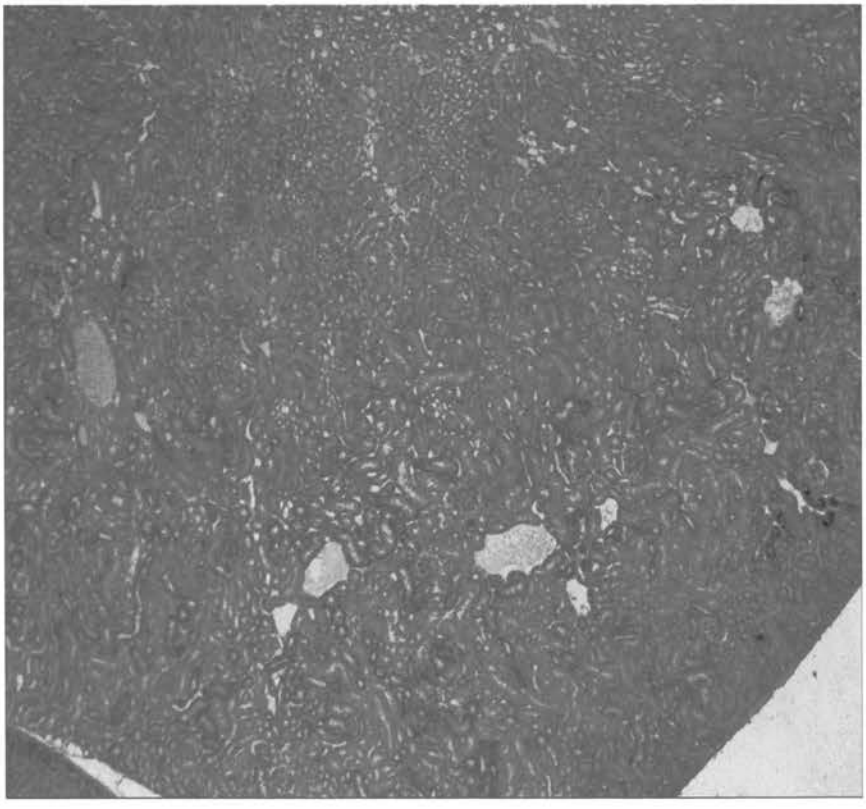
I.V. administration of Heme arginate upregulates functional HO-1 protein in murine renal tissue 24 hours post injection

A dose of 30mg/kg i.v. HA was found to reliably upregulate HO-1 protein predominantly in the tubules of the renal cortex rather than those of the medulla (Fig. 2.1). Western blotting performed to quantify this staining confirmed induction of HO-1 in renal tissue in a dose response pattern to a maximum at 30mg/kg (Fig. 2.2). Activity assays verified that HA treatment resulted in a significant induction of functional HO-1 protein, again following a dose response type pattern to maximum functional activity at a dose of 30mg/kg HA (Fig. 2.3. 66.7±13.3 vs. 480±100.7 bilirubin pmols/mg/hr; 3mg/kg vs 30mg/kg HA; $n=3$, $p<0.05$). Of note, a consistent "watershed" was observed between HO-1 induction within the cortical tubules and those of the outer stripe of the medulla. Images taken at high-power reveal the presence of a number of HO-1 positive interstitial cells in this area. Dual immunofluoresence with for HO-1 and the macrophage marker CD68 revealed these cells to be HO-1 positive macrophages (Fig 2.4). No such cells were observed in the outer stripe of the medulla of PBS treated animals.

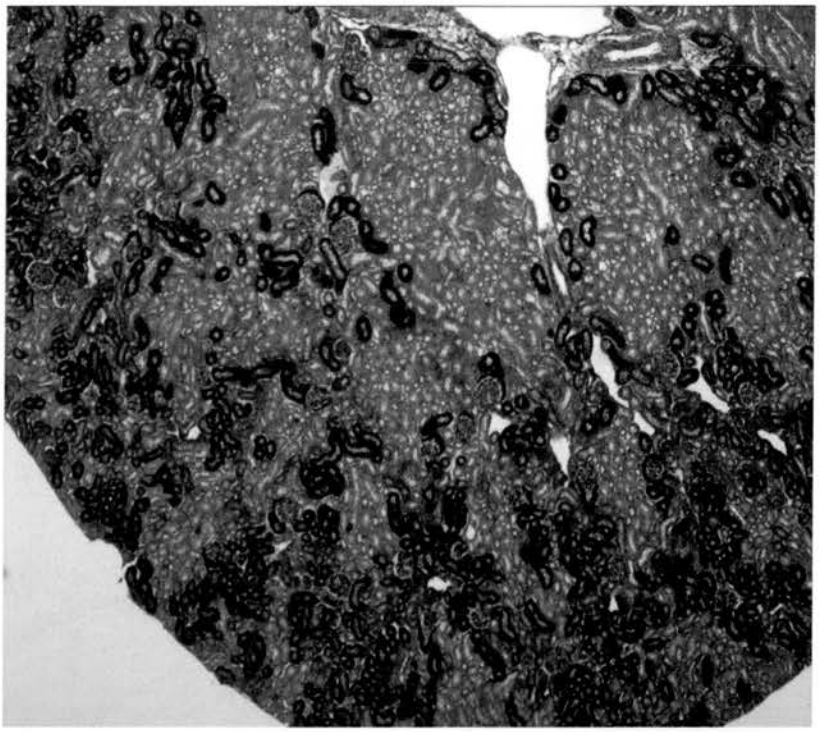
Fig 2.1



Isotype control (renal tissue) – Rabbit serum 1:1000 shows no positive staining



Normal saline – x20 objective image shows constitutive expression of HO-1 within tubules of the renal cortex



HA 30mg/kg – Tubules of the renal cortex stain strongly for HO-1. There is an abrupt "watershed" effect with minimal staining of tubules of the renal medulla.

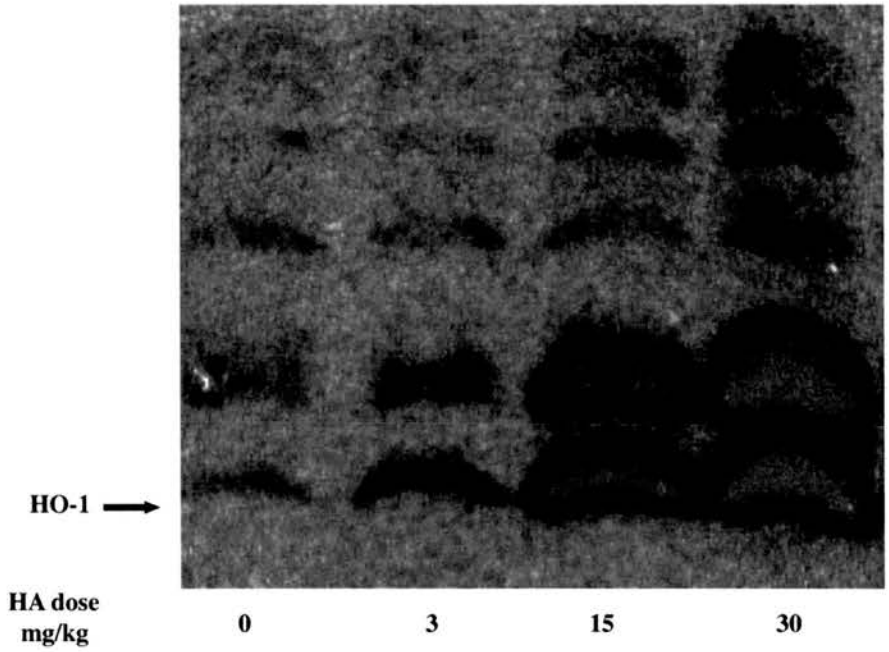


Fig 2.2 – HO-1 induction in renal tissue follows a dose response type relationship to a maximal induction at 30mg/kg. Constitutive expression of the enzyme is observed in control animals injected with normal saline (0mg/kg).

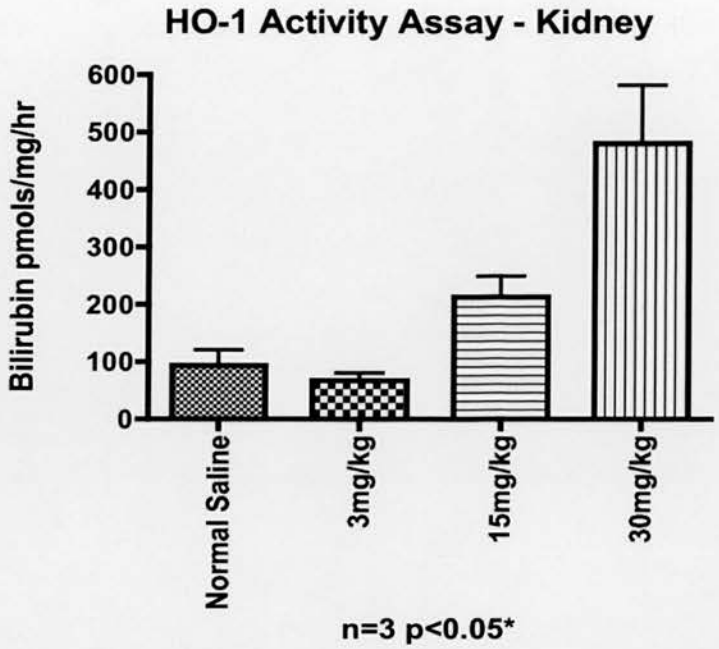
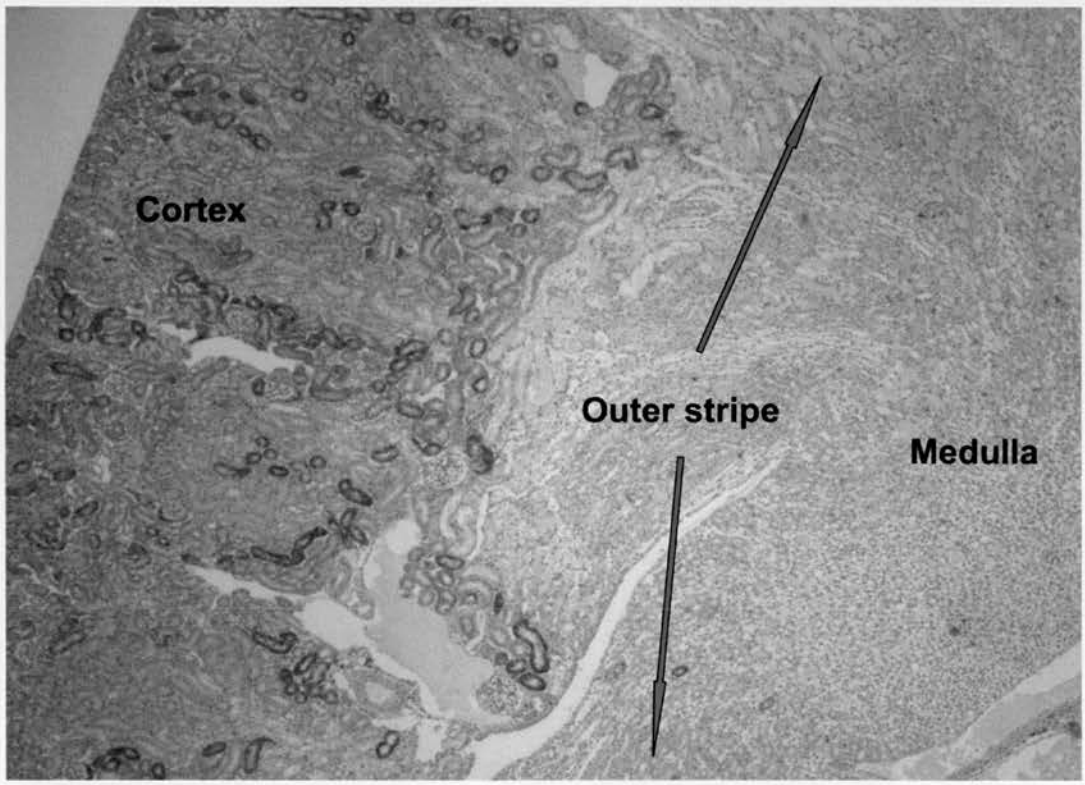
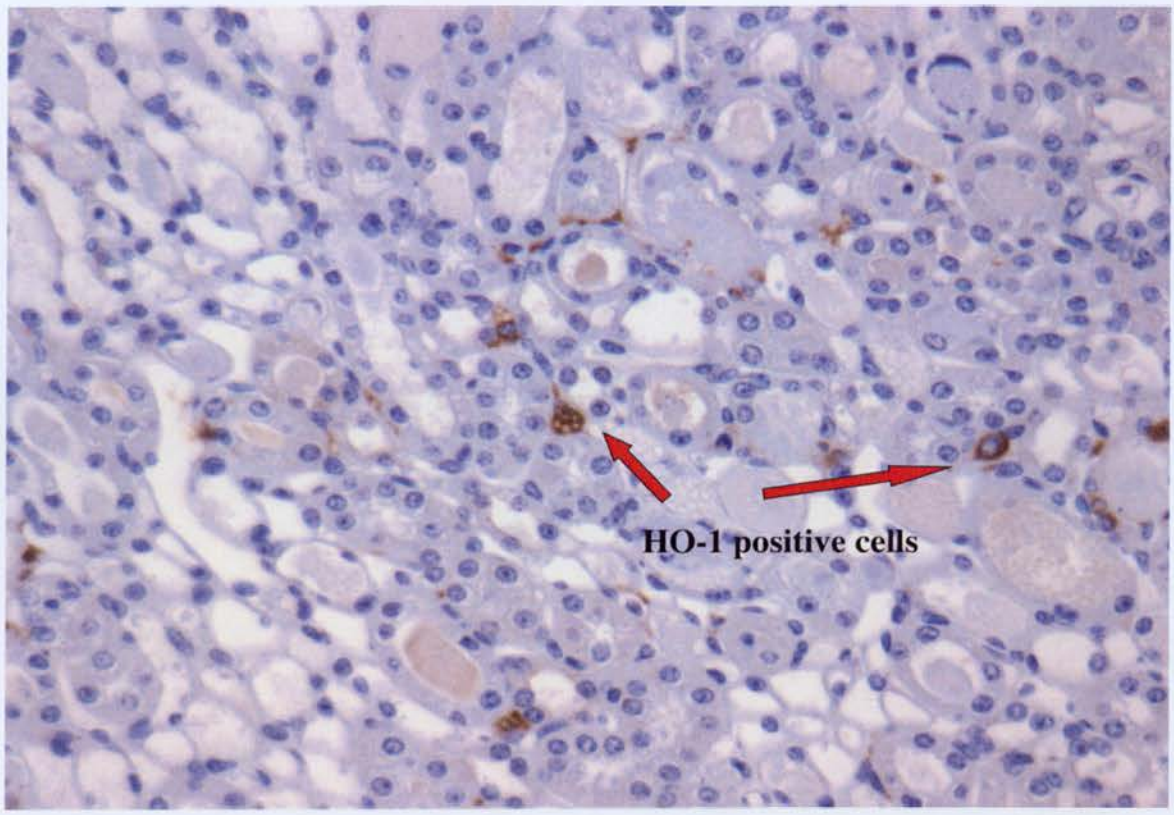


Fig 2.3 HA administration induces functional HO-1, with activity levels following a dose-response type relationship to a maximal activity at a dose of 30mg/kg (n=3, $p<0.05$).

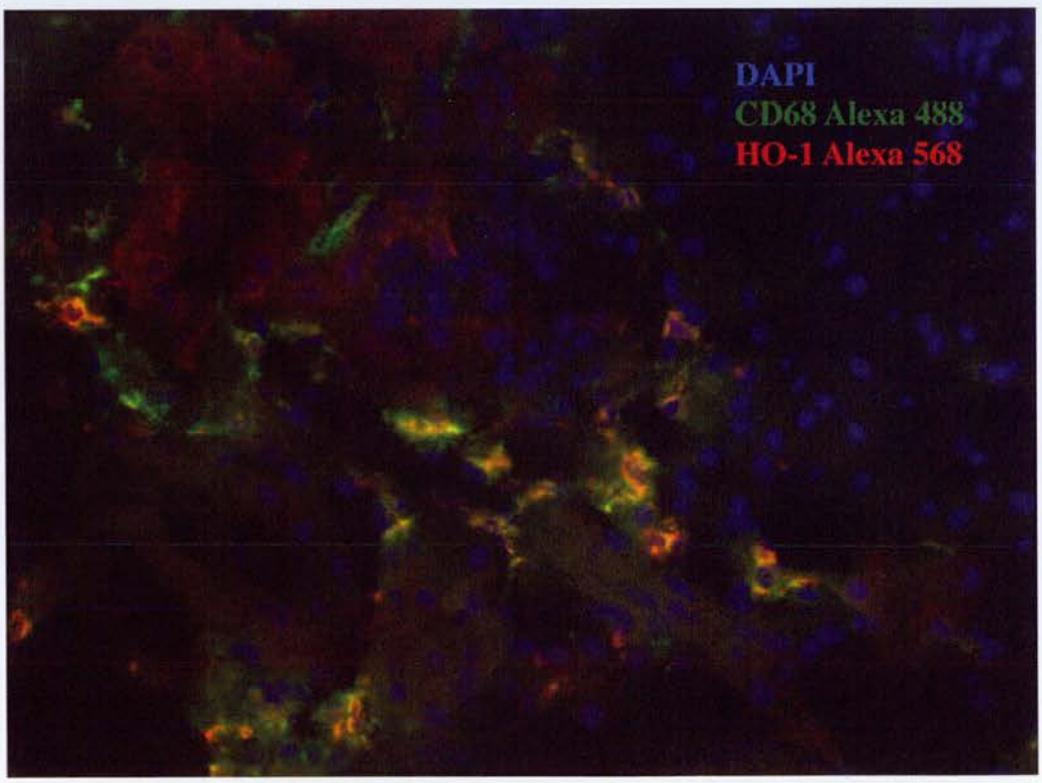
Fig 2.4



X20 objective. Following HA administration (30mg/kg). HO-1 expression is localised to the tubules of the cortex. HO-1 is not expressed by the tubules of the outer stripe of the medulla or the medulla itself



X200 objective. HO-1 positive cells are noted within the interstitial spaces, in association with tubules that do not express HO-1



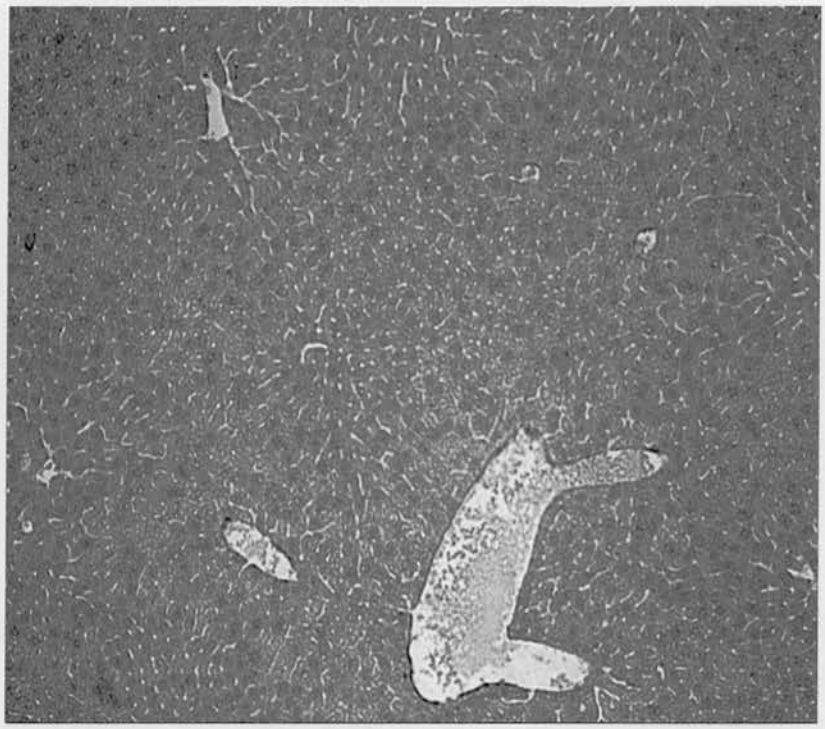
Dual immunofluorescence. CD68 labelled with Alexa 488 (Green), HO-1 labelled with Alexa 568 (red). Cells expressing both HO-1 and CD68 are highlighted in yellow. Implying that these cells are HO-1 positive macrophages.

I.V. Heme arginate upregulates functional HO-1 in murine liver 24 hours post administration

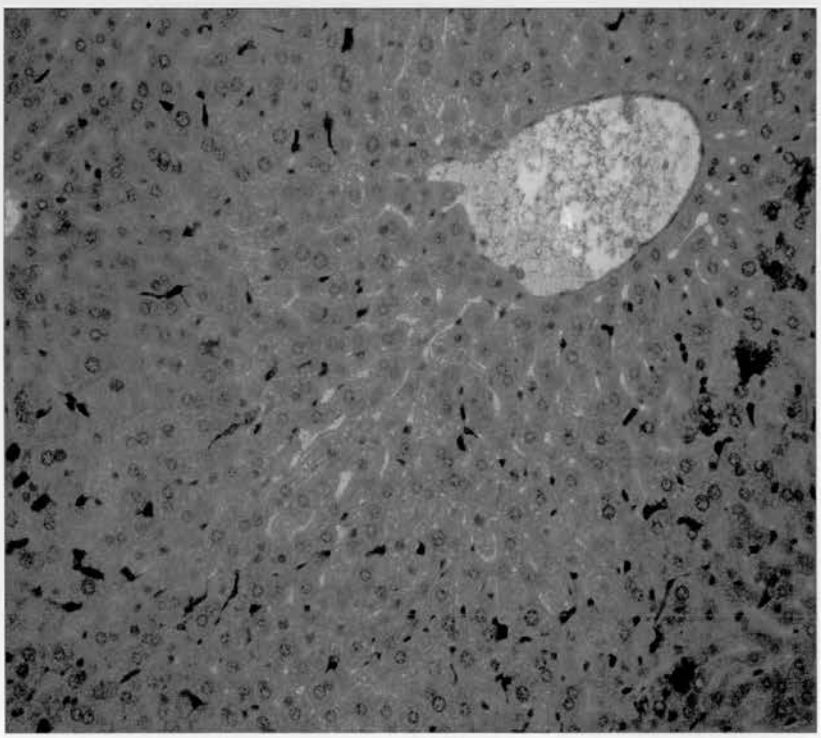
i.v Heme arginate administration resulted in upregulation of HO-1 in liver tissue 24 hours following administration (Fig 2.5). Quantification of this immunostaining by western blotting shows that, as in renal tissue, this induction followed a "dose response" type relationship. Maximum HO-1 expression was observed at a dose of 30mg/kg (Fig 2.6). Activity assays confirmed that there was functional upregulation of HO-1. Maximum activity was observed at a dose of 30mg/kg (93.3 ± 26.7 vs 386.7 ± 70.6 bilirubin pmols/mg/hr; Normal saline vs 30mg/kg HA; $n=3$, $p<0.05$) (Fig 2.7).

DAB immunohistochemistry suggested that HO-1 induction was concentrated in cells lying within the sinusoidal spaces. Dual immuofluorescence immunohistochemistry confirmed that these cells were positive for both CD68 and HO-1 indicating that they were HO-1 positive Kupffer cells (Fig 2.5). Such cells were not observed in PBS treated control animals. Sections from the spleens of HA treated animals also appear to show an increase in the numbers of HO-1 positive cells compared to sections from control animals. This increase occurred particularly within para-follicular areas, which are commonly populated by macrophages and T cells (Fig 2.8).

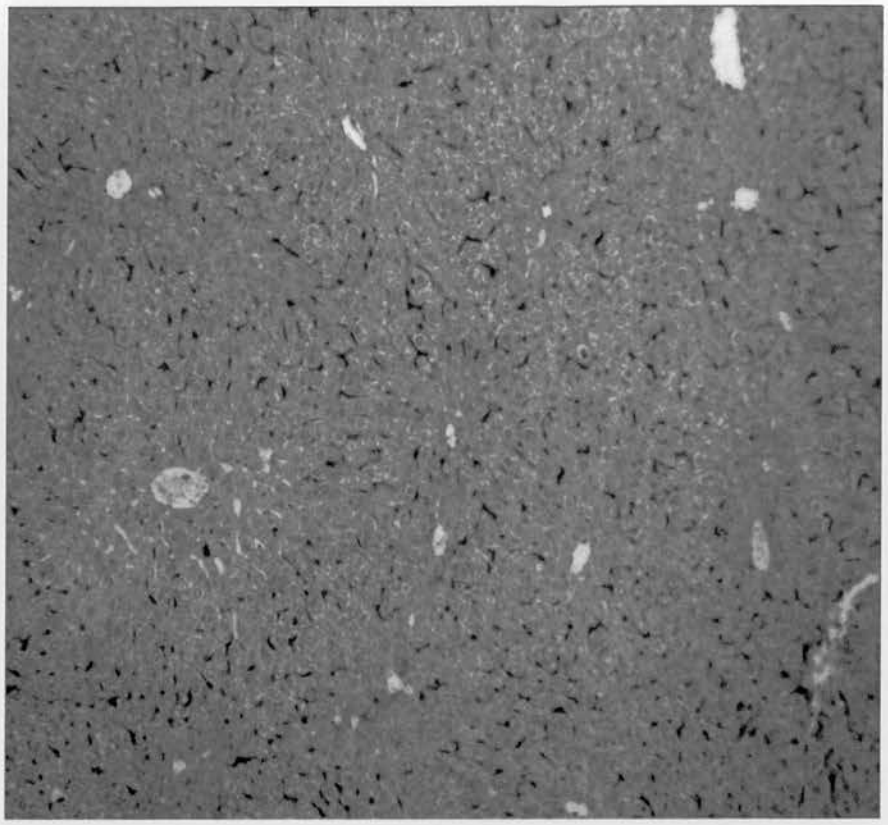
Fig 2.5



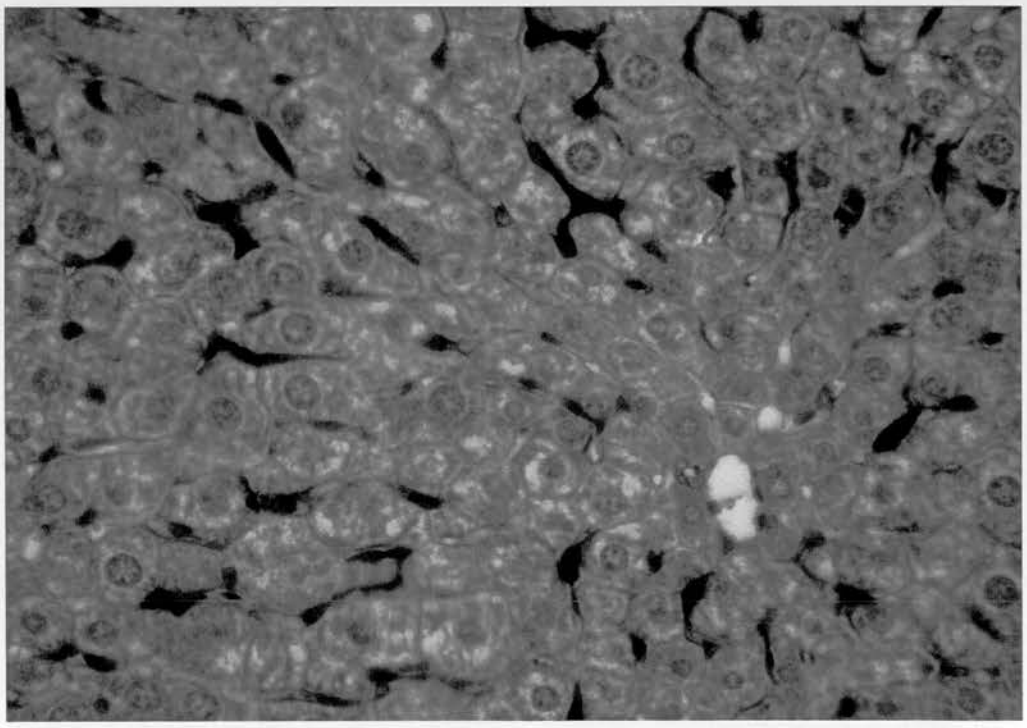
Isotype control – Rabbit serum 1:1000 shows no positive staining in liver tissue



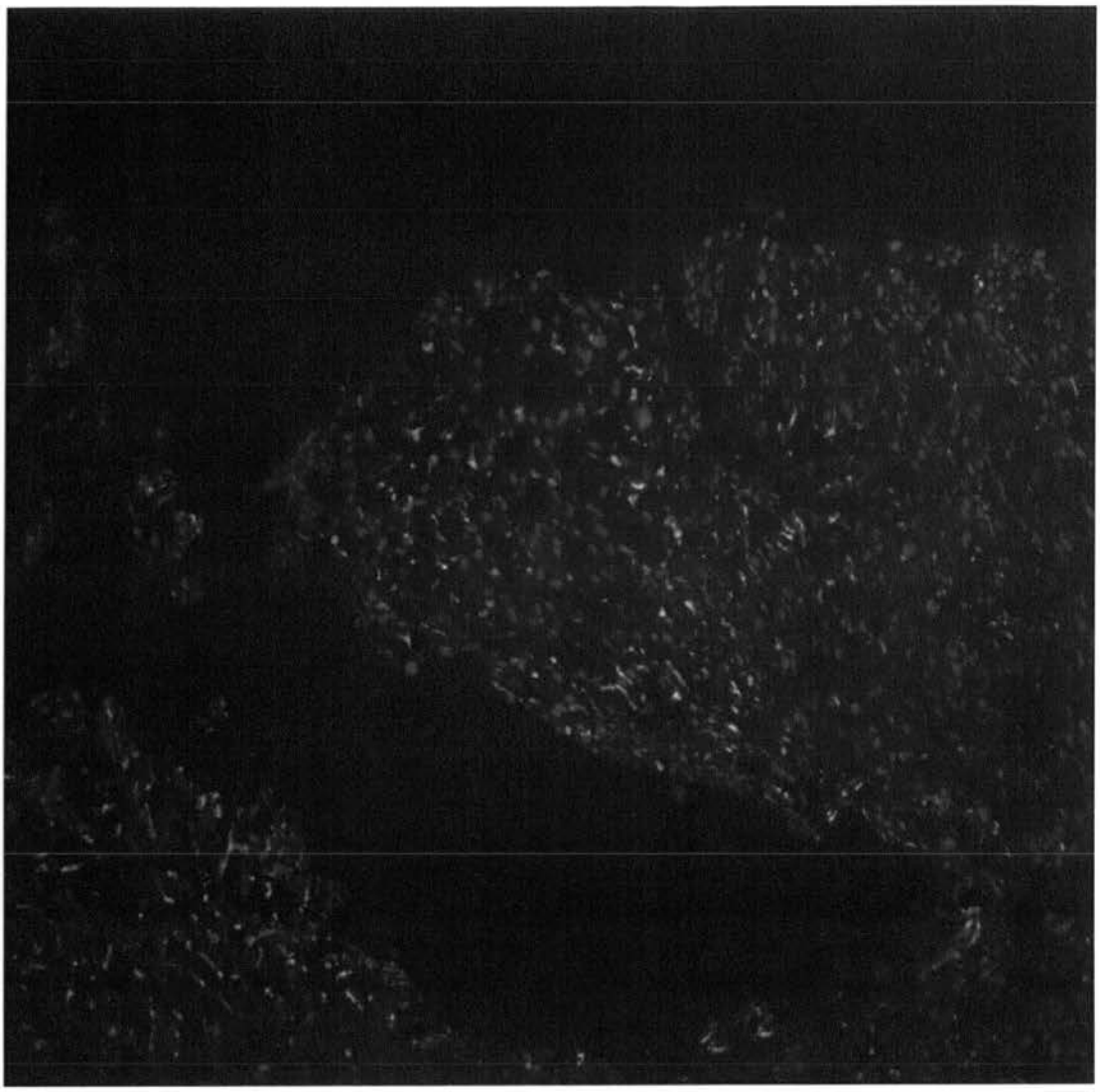
Normal saline – x20 objective image showing constitutive expression of HO-1 in cells lying within the sinusoidal spaces



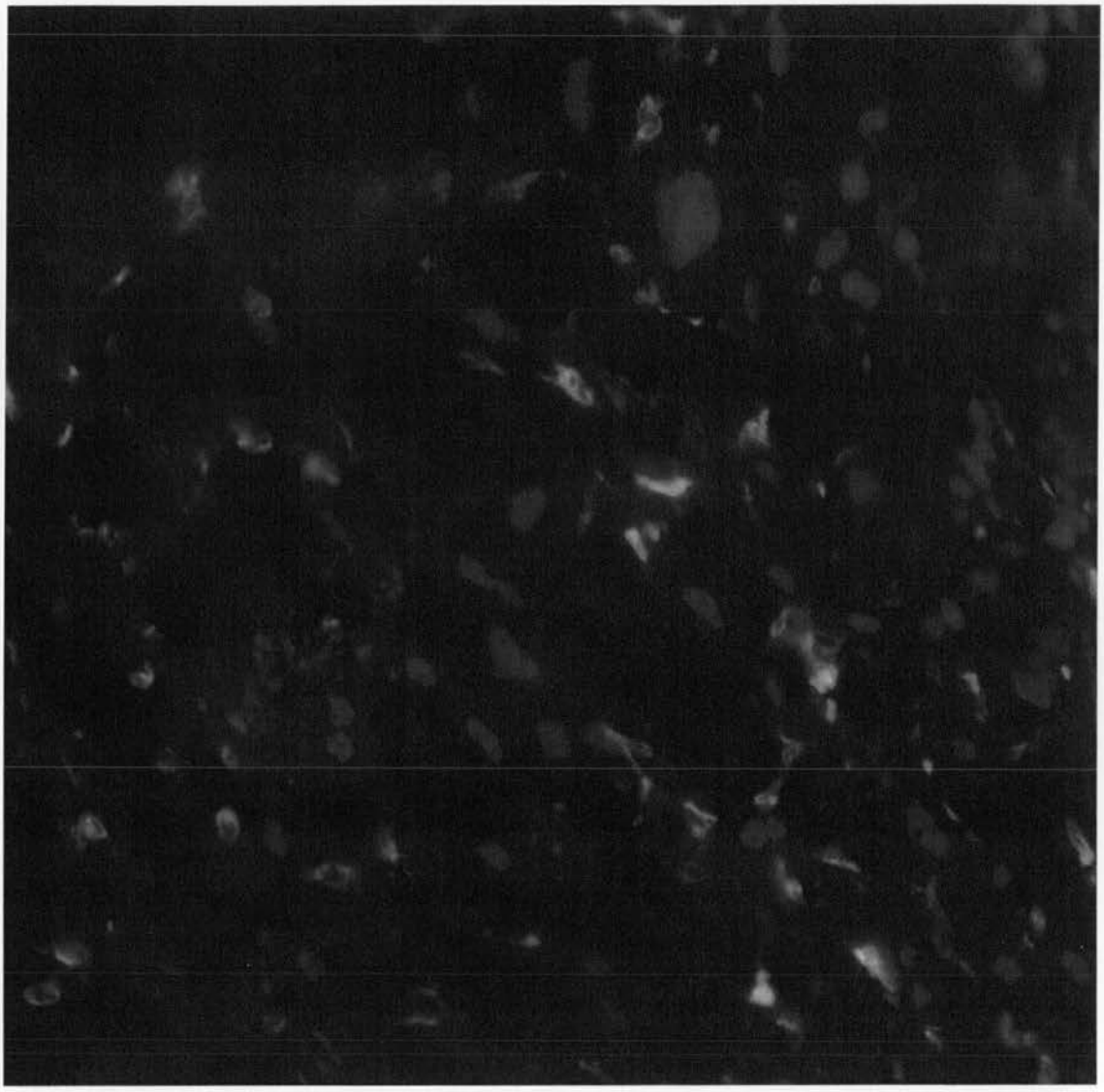
HA 30mg/kg – X20 objective showing increased expression of HO-1 in cells lying within the sinusoidal spaces



HA 30mg/kg – x200 objective showing expression of HO-1 by cells lying within the sinusoidal spaces. The hepatocytes themselves express little HO-1, although this may be visual artefact given their greater surface area



Dual immunohistochemistry. X20 objective from a mouse injected with 30mg/kg HA. CD68 labelled with Alexa 488 (green), HO-1 labelled with Alexa 568 (red). Counterstained with DAPI. Kuppfer cells expressing both HO-1 and CD68 are highlighted in yellow and are located in within the perisinusoidal spaces



Dual immunohistochemistry. X100 objective. 30mg/kg HA. Cells expressing HO-1 (red) and CD68 (green) are located within the peri-sinusoidal spaces

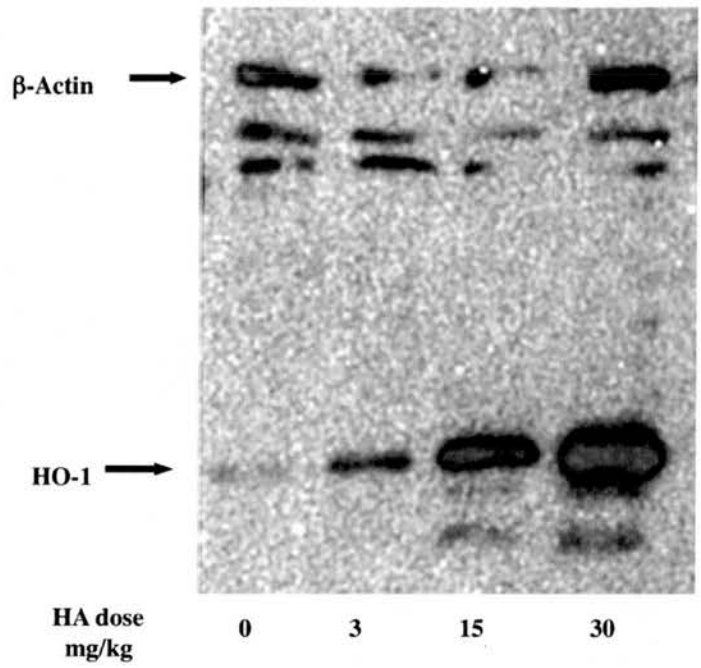


Fig 2.6 - HO-1 induction in liver follows a dose response type relationship to a maximal induction at 30mg/kg. Constitutive expression of the enzyme is observed in control animals injected with normal saline (0mg/kg).

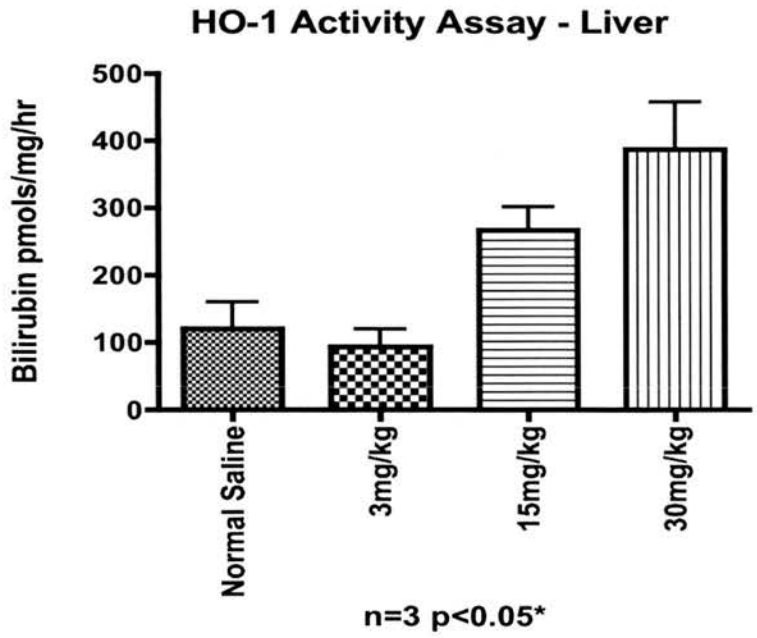
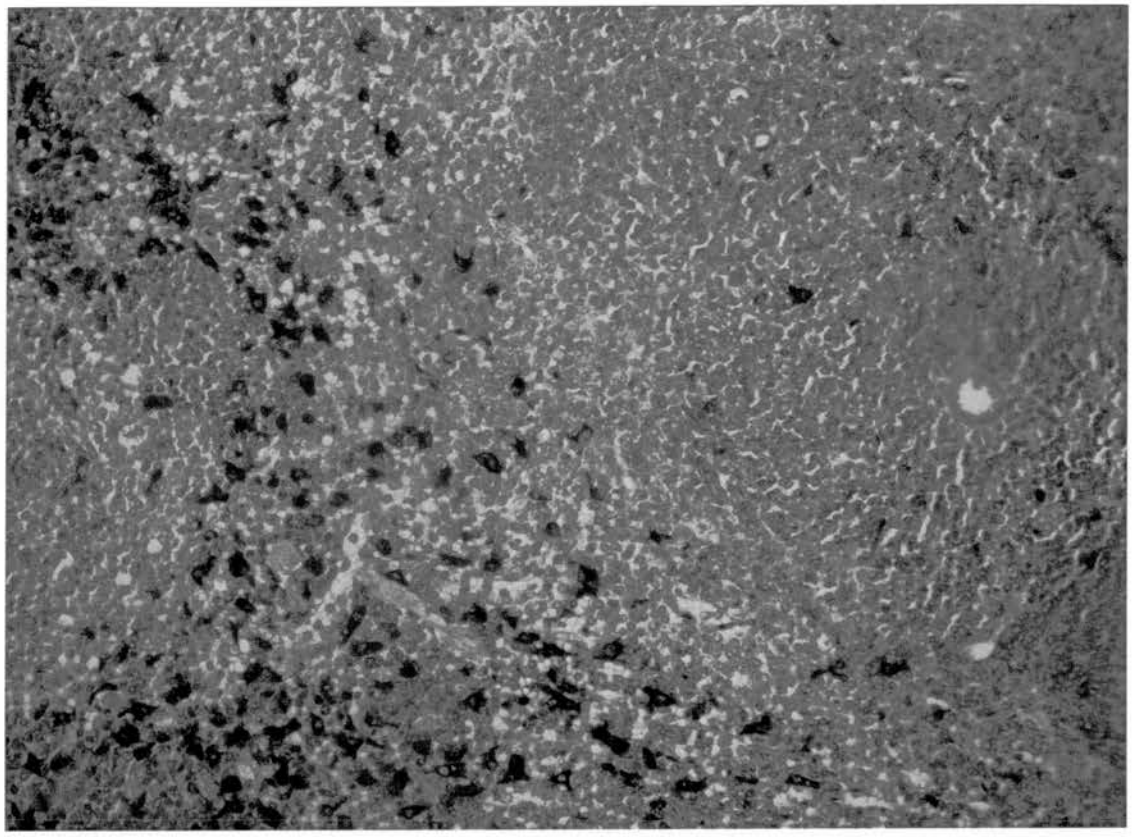
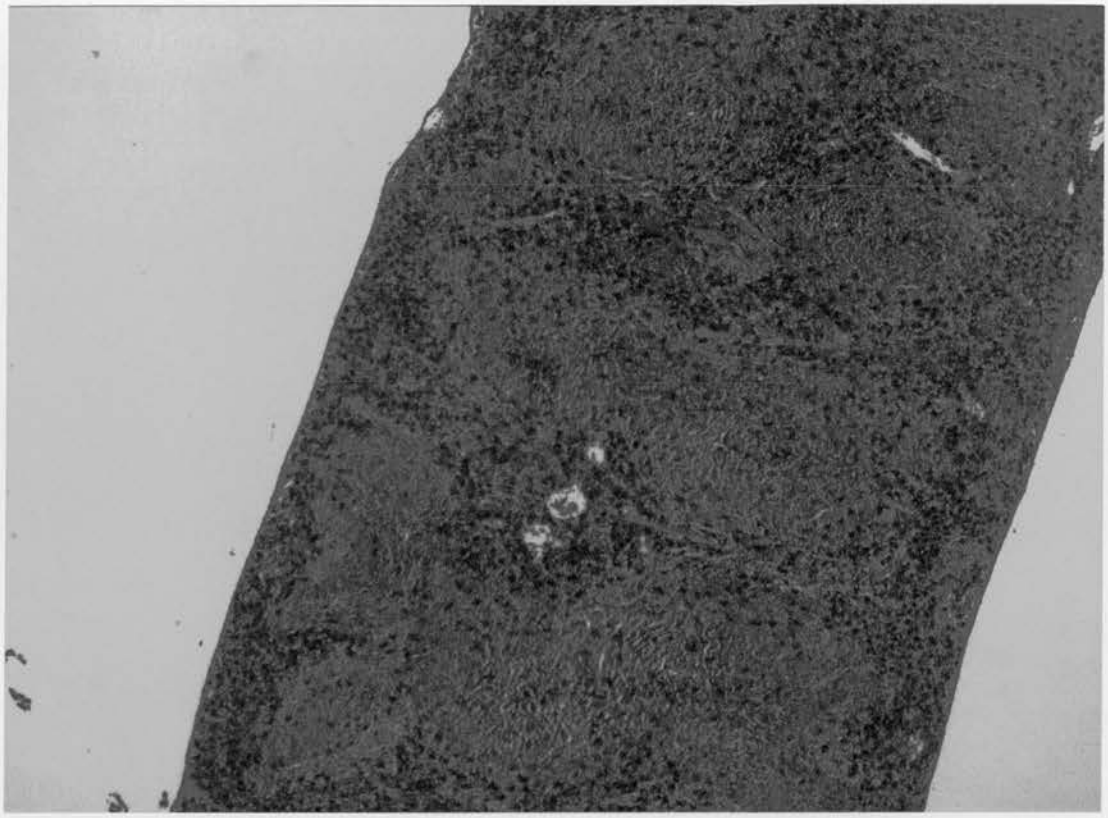


Fig 2.7 HA administration induces functional HO-1, with activity levels following a dose-response type relationship to a maximal activity at a dose of 30mg/kg (n=3, $p<0.05$).

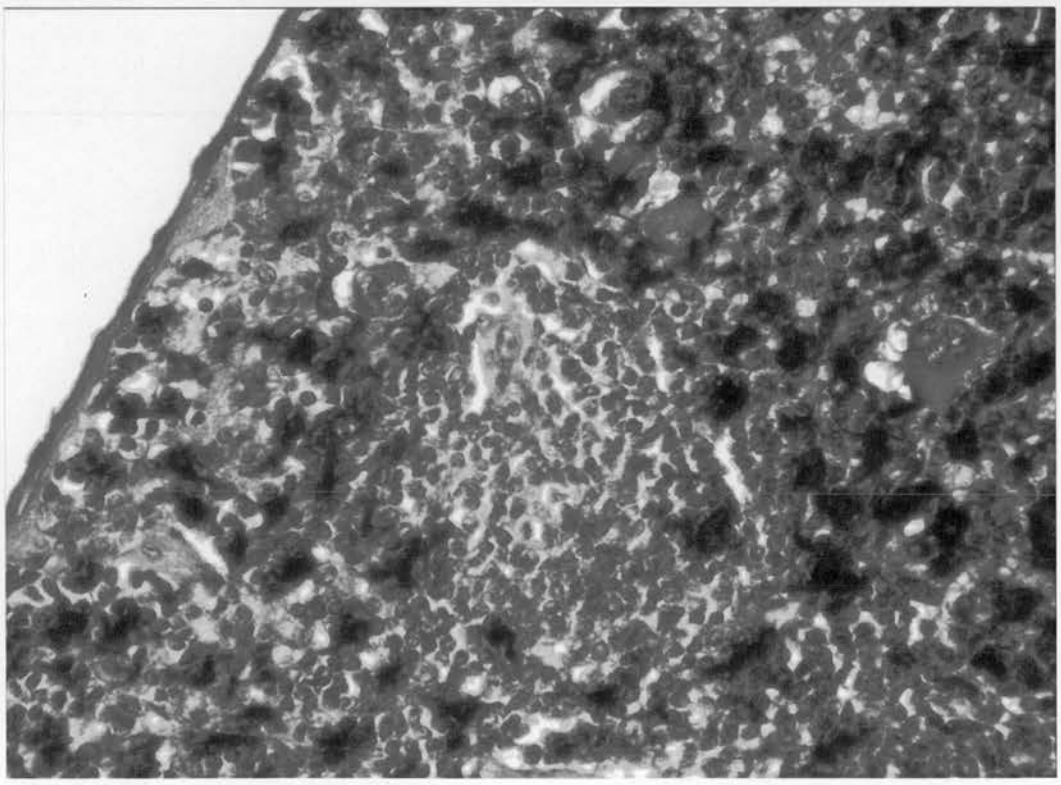
Fig 2.8

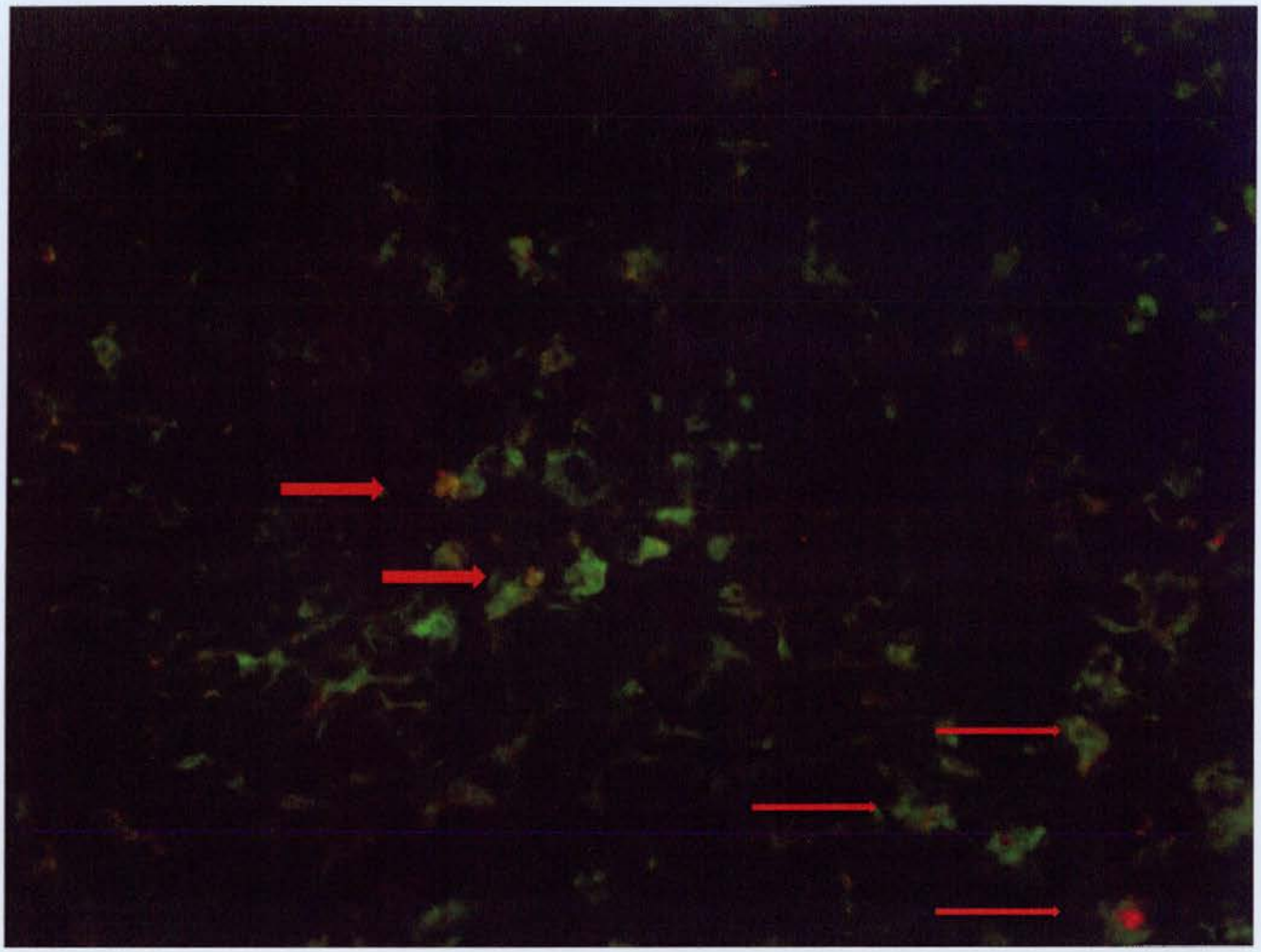


200μL PBS i.v. X100 objective of spleen showing constitutive expression of HO-1 by lymphoid cells located with para-follicular areas.



30 mg/kg HA x20 (above). There is increased expression of HO-1 by cells located within the splenic white pulp. X200 image (below)





Spleen X200 objective. Dual immunohistochemistry following 30mg/kg HA. Cells that co-express HO-1 (red) and CD68 (green) are highlighted by arrows.

Summary

This experimental data set demonstrates that:

1. HA treatment of mice induces functionally active HO-1 protein in both liver and kidney tissues. This follows a dose/response type relationship to a maximum induction at 30mg/kg.
2. Within kidney specimens, HO-1 induction is localised to tubules of the renal cortex. Tubules within the outer medulla do not express HO-1.
3. HO-1 is expressed by resident macrophages within the outer medulla of the kidney and Kupffer cells within the liver.
4. HA treatment is well tolerated by mice, with no apparent ill effects up to the maximum dose used (30mg/kg).

Discussion

Heme oxygenase-1 (HO-1) is a 32kDa enzyme that is induced by a variety of stimuli which include heme molecules, hypoxia, nitric oxide and endotoxin[160]. A growing body of evidence suggests that over-expression of HO-1 may be protective in animal models of IRI[168, 169, 177, 178] and transplantation[170, 171, 179]. The majority of such studies utilise metal protoporphryins as HO-1 inducing agents. These substances have numerous toxic side effects and are unstable at physiological pH, rendering them unsuitable for clinical use in humans. By contrast HA is a clinical grade heme protoporphyrin that is routinely used in the treatment of acute porphyria[180, 181]. The drug is generally well tolerated clinically, although a case report of anaphylaxis that was attributed to HA has been published[182]. The British National Formulary lists the principal side effects of HA as fever and thrombophlebitis at the injection site. A recent clinical study has also demonstrated that HA treatment upregulates HO-1 in healthy human volunteers, providing further validation for its potential use in clinical trials[183]. Only a few in vivo studies that utilise HA have been published, but these

appear to provide further evidence of the protective benefits of this drug. HA was shown to improve liver microcirculation and mediated an anti-inflammatory cytokine response in a rat model of simulated haemorrhagic shock[184]. Previous work from our own group has also shown that HA pre-treatment reduces structural injury and preserves renal function in aged mice subjected to renal IRI[50].

The data set presented in this chapter confirms that HA treatment upregulates functional HO-1 in a dose response pattern within murine liver and kidney tissue. Doses up to 30 mg/kg were apparently well tolerated by the animals, with no obvious adverse side effects or deaths within either the treatment or control groups. 30mg/kg was selected for the purposes of further in vivo experimentation given the maximal induction of HO-1 at this dosage. The patterns of HO-1 expression that were observed in renal tissue are of particular interest. An abrupt "watershed" in the staining was consistently noted. The tubules of the renal cortex strongly expressed HO-1, in contrast to those of the medulla and the outer stripe of the medulla (OSOM), which did not. Potential explanations for this pattern of HO-1 expression might be found in renal vascular physiology. Tubules in the renal cortex are exposed to higher initial concentrations of HA within the vascular supply. They receive their blood supply from the proximal part of the efferent arterioles that form immediately after the circulation of blood through the glomeruli. There may therefore be an insufficient concentration of HA remaining within the more distal efferent arterioles that supply the tubules of the OSOM to enable significant HO-1 induction. At high magnification however, we did observe a population of HO-1 positive cells in the OSOM following HA administration. These cells were found in close physical proximity to the tubules of this region that did not express HO-1. Co-expression of CD68 and HO-1 by immunofluorescence indicates that these cells were HO-1 positive macrophages. Should HA administration prove to be protective in these models, the absence of HO-

1 positivity in the tubules of the OSOM would suggest that direct tubular expression of HO-1 might play a less important role within that mechanism than other factors.

Some similarities were noted between the HO-1 staining pattern observed in the liver following HA administration and those in the kidney. Numerous HO-1 positive cells were identified within liver sinusoids, whereas hepatocytes by comparison expressed apparently little HO-1. Dual immunohistochemistry for CD68 and HO-1 confirmed that these cells were in fact HO-1 positive Kupffer cells (tissue resident macrophages). It is worth noting that the concentration of DAB in macrophages within the relatively small space represented by a single macrophage (in comparison to the much larger hepatocytes) may give the appearance of more intense HO-1 expression. The hepatocytes themselves may in fact contain increased absolute levels of the enzyme, yet distributed over a much wider area, giving the impression of less intense HO-1 staining. The findings in this study are in keeping with those of other authors. Devey et al have reported Kupffer cells to be the principal site of HO-1 expression in the liver[267]. These authors selectively depleted Kupffer cells and circulating monocytes in a model of hepatic ischaemia reperfusion injury that was based upon the CD11b DTR mouse. They noted that deletion of these cells rendered mice highly susceptible to hepatic IRI and concluded that tissue resident macrophages protected the liver from IRI via a HO-1 dependent mechanism. Such findings may be of relevance within our own models of native kidney IRI, and isograft transplantation, given the absence of HO-1 expression within the tubules of the outer medulla, and the observation that HO-1 positive macrophages are present at this site 24 hours after HA administration.
The sections from the spleens of HA-treated mice showed a pattern of HO-1 expression that was predominantly within the parafollicular areas. Some of these cells were also found to be co-positive for CD68 and HO-1, suggesting HO-1 expression by splenic macrophages, in keeping with the findings in liver and renal tissue. Given

the para-follicular location of the HO-1 positive cells, it is possible that some of the expression may also be by T-cells. It is established that HO-1 induction has an immunosuppressant action[268] and has been shown to inhibit the differentiation and proliferation of cytotoxic T cells, whilst prolonging the survival of cardiac allografts[269]. Further immunophenotyping to clarify precisely which cell subtypes express HO-1 within the spleen may be of value, particularly with reference to the potential use of this drug in renal allograft transplantation.

This study consistently demonstrates HO-1 expression by macrophages in liver, renal and splenic tissue following HA administration. However, it was less clear whether HO-1 was expressed directly by endothelial cells within the microvasculature. The pattern of HO-1 positivity within the liver and the outer medulla of the kidney was not consistent with HO-1 expression by endothelial cells in these organs. This observation is in keeping with the published literature. Whilst HO-1 induction is reported in cultured endothelial cells in vitro [270, 271], localisation of HO-1 expression to the endothelium in vivo is less consistently observed[272, 273]. This may reflect differences in the HO-1 inducing agents used between studies, as well as a variety of other factors. For example, Ali et al[235] noted that HO-1 expression by vascular endothelium following statin administration was enhanced following exposure to laminar sheer stress and impaired by disturbed flow. Such variables may account for some of the differences within published accounts. It is also possible that immunohistochemistry may not be sufficiently sensitive to highlight HO-1 expression by the endothelial cells of the peri-tubular capillary network. These cells are of low volume and have a flattened shape; patchy HO-1 positivity distributed throughout such a cell might therefore be rather indistinct. Laser micro dissection techniques or flow cytometry may help to clarify whether these cells do upregulate HO-1 in response to HA administration. However, if HO-1 is not expressed by the tubules of this region, it is plausible that there may similarly be little or no expression of HO-1 by the

microvasculature in the same site. The physiological explanation suggested earlier, with reference to the tubules of the OSOM may also be relevant in the case of endothelial cells. Whilst preservation of the microvasculature may ultimately prove to be critical to HA mediated protection in IRI, that protection need not necessarily be via direct expression of the enzyme by the endothelium. Rather, the effects of the systemic induction of HO-1 upon the preservation of renal blood flow and improvements in renal haemodynamics may prove to be more important than the limitation of endothelial cell loss. Renal haemodynamics have been shown to be impaired following renal ischaemia in HO-1 knockout mice when compared with wild type controls[274]. Some authors have also noted a significant enlargement of the vascular diameter and an increase in capillary blood flow in transplanted kidneys following treatment with the HO-1 inducer hemin[199]. It would be interesting to evaluate similar parameters in mice following HO-1 induction by HA through the use of intra-vital microscopy or a similar imaging technique. The alterations in renal haemodynamics that have been described may be related to CO production as a bi-product of haem catabolism by HO-1. CO is thought to mediate some of the protective benefits of HO-1 through inhibition of platelet aggregation[275] and increased vasodilatation[173]. Both of these phenomena would enhance organ perfusion and therefore reduce ongoing ischaemic damage. Attempts were made to measure carboxyhaemoglobin levels in the blood of these HA treated animals but unfortunately proved unsuccessful. This was probably due to inadequate sensitivity of the clinical blood gas analyser used. The HO-1 activity in the dose response experiments outlined in this chapter was measured in units of pg of bilirubin formed per mg protein per hour i.e. in minute quantities. However, given that this series of experiments has shown increased functional activity of HO-1 and a measured increase in bilirubin levels, it is reasonable to assume that there will be a similar increase in CO levels within the tissues.

Chapter 3: In vivo models of native kidney IRI and renal isograft transplantation

Introduction

Data presented In the first chapter of this thesis established that HA pre-treatment confers protective benefits of upon MCEC-1 cells that were exposed to conditions which replicate those encountered during IRI in vivo. In chapter 2 it was shown that HA treatment of mice upregulates functional HO-1 protein in both liver and kidney specimens and that HO-1 expression within the kidney was localised to tubules of the renal cortex. The tubules of the medulla, and the outer stripe of the medulla did not express HO-1 following HA administration. HO-1 positive interstitial macrophages were identified within this region, in association with these HO-1 negative tubules. The next phase of experimental work involved determining whether HA treatment can protect renal structure and function in animal models of native kidney IRI and renal isograft transplantation. Our in house model of native kidney IRI is well-established[50, 67] and allows for the measurement of postoperative renal function (serum creatinine levels) in conjunction with quantification of structural injury (ATN score). Our model of murine renal isograft transplantation is stable and reproducible with a good technical success rate[219, 276]. The organ recipient within this model retains a functioning kidney and therefore measurements of renal function are not relevant. This model was used to compare HA treatment of the organ donor with treatment of the organ recipient. Particular reference was given to the structural integrity of the microvasculature as measured by CD31 immunohistochemistry in both models. The numbers of HO-1 positive interstitial cells within the outer stripe of the medulla were quantified prior to and following injury within each system.

Materials and methods

Murine model of ischaemia reperfusion injury

All mice were males aged 6-8/weeks old, on inbred FVB/nj strain background purchased from Harlan (UK). Procedures were performed under Home Office guidelines. Animals received pre-treatment 24 hours prior to surgery with either 200μl PBS or 200μl of a solution containing 30mg/kg Heme arginate (Normosang; Leiras Oy Pharmaceuticals, Finland) diluted in PBS. HA solution was administered via penile vein injection under brief inhalational isofluorane anaesthesia. Anaesthesia was induced prior to ischaemia/reperfusion injury surgery using Ketamine and Metatomidine via the intraperitoneal route, with Buprenorphine analgesia subcutaneously. Via a midline laparotomy a right nephrectomy was performed and the left renal pedicle identified and clipped using an atraumatic clamp for 20 minutes. The right nephrectomy specimen was termed "day 0" kidney and was analysed for HO-1 expression. During the ischaemic period body temperature was maintained at 35°C using a heating blanket with homeostatic control (Harvard Apparatus, Boston MA) via a rectal temperature probe. The clamp was then removed, the peritoneum closed with 5/0 suture, the skin closed with clips, and anaesthesia reversed using atipamazole. 1ml sterile saline was administered subcutaneously prior to and post surgery. The animals were maintained in an incubator overnight at (27°C), with further s/c saline given the next morning. Blood and tissue samples were obtained at 24 hours post surgery under terminal anaesthesia. The post ischaemic kidney was termed "day 1" kidney. The experimental procedure is summarised in Fig 3.1.

Assessment of renal function

Plasma samples were prepared from whole blood, and measures of serum creatinine were obtained (in μMol/L) by the Jaffe method (Alpha Laboratories Ltd., UK) on a Cobas Fara Centrifugal Analyser (Roche Diagnostics, UK) according to the manufacturer's instructions.

Model of renal isograft transplantation

All mice were males aged 6-8 weeks old on inbred FVB/nj strain background (Harlan, UK). Procedures were performed under Home Office guidelines. 24 hours prior to surgery, the mice were anaesthetised transiently with inhalational isofluorane and treated with 200mL PBS or 30mg/kg Heme arginate (Normosang, Leiras Oy Pharmaceuticals, Finland) via penile vein injection. Both donor and recipient mice were administered heparin prior to the transplant surgery. Anaesthesia was induced by intraperitoneal ketamine (70mg/kg) and medetomidine (1.0mg/kg). Subcutaneous buprenorphine was administered as an analgesic (0.06mg/kg). Following a midline laparotomy, the left kidney was isolated on its vascular pedicle and the ureter tied off and divided close to the bladder. The vena cava and cranial aorta were then tied off. The caudal aorta was divided and the kidney was perfused with University of Wisconsin (UW) solution maintained at 4°C. The nephrectomy was completed by dividing the renal vein, cranial aorta and ureter. The donor kidney was stored in UW solution at 4°C. Recipient mice underwent a right nephrectomy. Parallel sections of vena cava and aorta caudal to the left renal pedicle were isolated with arterial clamps. The renal vein was anastomosed with the vena cava and the renal artery with the aorta with 11/0 sutures such that the donor kidney lay on the right side. Following removal of the clamp, the ureter was anastomosed to the bladder using 9/0 sutures and the peritoneum was then closed with 5/0 suture. The skin was closed with clips. Anaesthesia was reversed using atipamazole (2mg/kg). Prior and post-surgery, 1 ml

subcutaneous (s/c) sterile saline was administered. The mice were kept in an incubator overnight (at 27°C) followed by further s/c saline in the morning. Tissue was harvested 24 hours after surgery following termination by cervical dislocation. The animals were kept under close observation post-operatively and culled if recovery was slow. The technical success rate of the model was 75%, defined as survival to the end of the experiment and the presence of a viable kidney on completion. The experimental procedure is summarized in Fig 3.2.

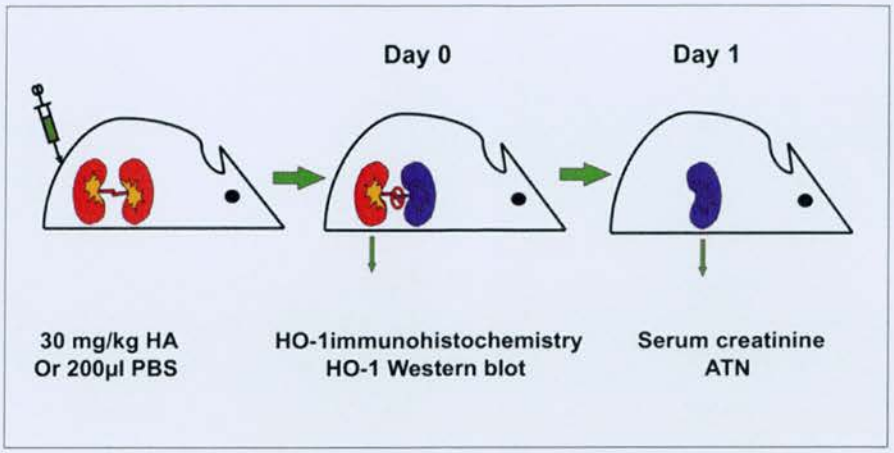
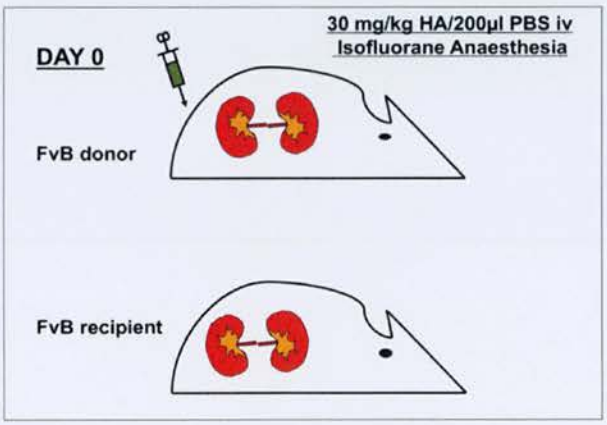
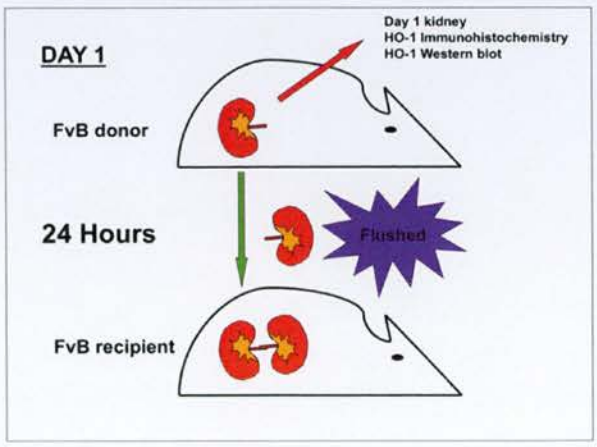


Fig 3.1 Overview of IRI experiment. Green arrows represent 24 hour time periods. On day 0 the left kidney is subjected to 20 mins reversible warm ischaemia following a right nephrectomy (day 0 kidney). 24 hours post surgery, blood and tissue samples (day 1 kidney) were obtained under terminal anaesthesia.

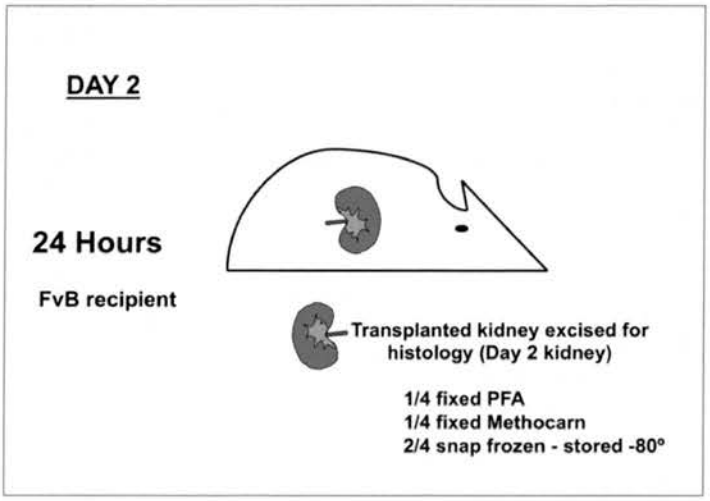
Fig 3.2



Day 0: 6 week-old FvB mice are injected with 30mg/kg HA or 200µL PBS i.v. These animals are either used as donor or recipient animals in subsequent transplant experiments.



Day 1: 24 hours after pre-treatment of either the donor or recipient, a kidney is retrieved from the organ donor and implanted into an FvB recipient animal. The contra-lateral kidney (day 1 kidney) from the donor is analysed to confirm HO-1 up-regulation. In the recipient treated group, the nephrectomy specimen from the recipient taken prior to implantation of the transplanted kidney is taken as the day 1 control.



Day 2: After 24 hours transplanted animals are sacrificed and the post-transplant kidney (day 2 kidney) is retrieved for analysis.

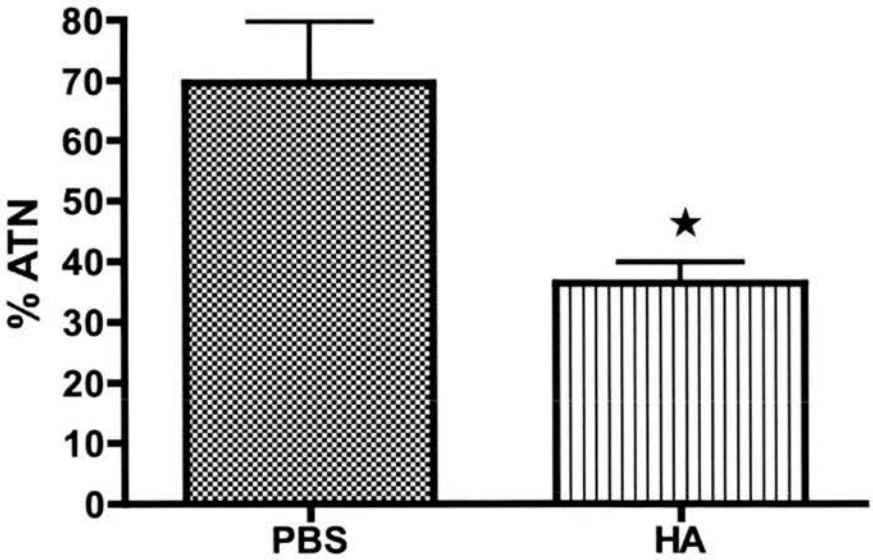


FIG 3.3: Pre-treatment with HA significantly protects renal structure against renal IRI (n=8 vs 5, $p<0.05$).

Immunohistochemistry and quantification of CD31 staining

Whole kidneys were cut longitudinally and either snap frozen in liquid nitrogen, fixed in methyl Carnoy's solution (60% methanol, 30% chloroform and 10% acetic acid) or 4% paraformaldehyde prior to embedding in paraffin. 4μm tissue sections were cut and stained with haematoxylin and eosin for assessment of medullary tubular necrosis. For assessment of CD31 expression, formalin fixed sections were treated in protease K solution (12.5mg/100ml PBS preheated to 37°C, for 20 minutes at 37°C, Sigma-Aldrich, Gillingham, UK) and incubated with rat monoclonal antibody (1:10 dilution, 4°C overnight; BD Biosciences Pharmingen, Oxford, UK). This was followed by biotinylated rabbit anti-rat primary antibody (DakoCytomation, Gostrup, Denmark). For CD31 immunohistochemistry, 2 drops of HRP rabbit EnVision reagent (Dako UK limited, Cambridgeshire UK) were added for 30 minutes prior to DAB counterstaining. HO-1 expression was assessed following antigen retrieval in citrate buffer (10mM sodium citrate, 0.05% tween. Microwaved at 800W for 3 x 5 min). Sections were then incubated with rabbit anti mouse polyclonal antibody (1/500 dilution; 4°C overnight; Stressgen Biotechnologies, Vancouver, Canada) Renal macrophages (MΦ) were identified by immunostaining for the tissue MΦ marker F4/80. Methyl Carnoy's fixed tissue was deparaffinized in xylene, rehydrated and blocked using 3% H_2O_2 prior to incubation with rat anti-F4/80 monoclonal antibody (1/250 dilution; Caltag Laboratories, Northampton, UK). Infiltrating neutrophils were identified by nuclear morphology combined with immunostaining for the Gr1 (Ly6c/g) antigen using Rat anti-Gr1 monoclonal antibody (1/350 dilution; Cambridge BioScience, UK). All rat anti-mouse antibodies were incubated at 4°C overnight with subsequent incubation with mouse-adsorbed biotinylated rabbit anti-rat IgG (1/300 dilution; Vector Laboratories, Peterborough, UK) at room temperature (RT) for 30 minutes. After washing sections were incubated with Vectastain ABC Elite reagent

(Vector Laboratories, Peterborough, UK) for 30 minutes at RT, prior to washing and staining with diaminobenzidine (DAB) (Dako UK, Cambridgeshire, UK). Counterstaining was performed with hematoxylin prior to dehydration and mounting. In all cases appropriate isotype antibodies were used as negative controls. MΦ and neutrophils were identified by morphology and staining pattern, and expressed as mean cells per x400 microscope field, with 3 fields being assessed per section. Tubules within the outer stripe of the outer medulla (OSOM) were photographed using a Leica DC300 digital camera (x50) on a Leica DMLB microscope and tubules counted as viable and necrotic tubules on the basis of nuclear morphology and integrity of the epithelial cell layer using ImageJ software (Cell_counter plugin; ImageJ 1.36b; National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/Java1.5.0_13). CD31 immunostaining was quantified from x400 microscope field images, with 3 fields being captured per section. Subsequent analysis of the area of DAB positive staining was performed using Colour Range tool on Photoshop CS3 Extended (Version 10.0.1; Adobe Systems Europe, Uxbridge, UK). Results were expressed as CD31 positivity as a percentage of total area. All slides and images were assigned a number to ensure that subsequent analysis was performed in a blinded fashion, without knowledge of the treatment groups. In the case of the H&E stained slides used for assessment of tubular necrosis, the blinding process was performed by a third party in a randomised fashion.

Results

Heme arginate pre-treatment protects renal structure and function following native kidney IRI

Histological injury was quantified in the outer medulla 24hr following IRI by counting the percentage of necrotic tubules in high-power fields (X200 objective). Fields were quantified until the whole of the outer medulla represented in the sections

had been visualised. Pre-treatment with HA offered significant protection against acute tubular necrosis when compared with PBS treated animals (Fig 3.3, see page 92. 69.6 ±10.2% vs. 36.5 ± 3.5 %ATN PBS vs. HA; n=8 vs.5, $p<0.05$). A macroscopic comparison of a typical post IRI kidney from each group is represented in Fig 3.4. PBS control kidneys were noticeably more oedematous and haemorrhagic compared with those from the HA treated group. Typical histological sections from each group are compared in Fig 3.5. The differences in n number between the two groups was due to a lack of representation of OSOM within the plane of section in 3 HA treated animals. HA treated animals also had significant preservation of renal function after surgery compared with PBS treated controls (Fig 3.6. Serum creatinine 132 ± 44.4 vs 78 ± 24.3µmol/l PBS vs. HA; n=8, $p<0.05$).

Pre-treatment of the organ donor with Heme arginate protects renal structure in a model of murine renal isograft transplantation

Histological injury was quantified within the outer medulla 24 hours after isograft transplantation as previously described. Pre-treatment of donor animals with HA significantly protected the transplanted kidney against acute tubular necrosis when compared with PBS treated controls (Fig 3.7. 54.1 ± 9.4% vs. 23.46 ± 7.8% ATN PBS vs. HA; n=10, $p<0.05$).

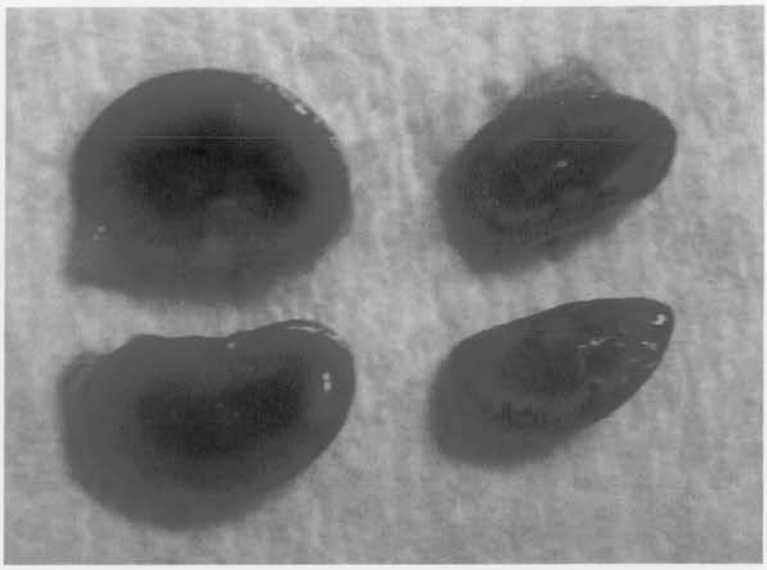


Fig 3.4: Typical macroscopic appearance of post IRI kidneys. Specimens from PBS treated animals were noticeably more haemorrhagic and oedematous than those of HA treated animals.

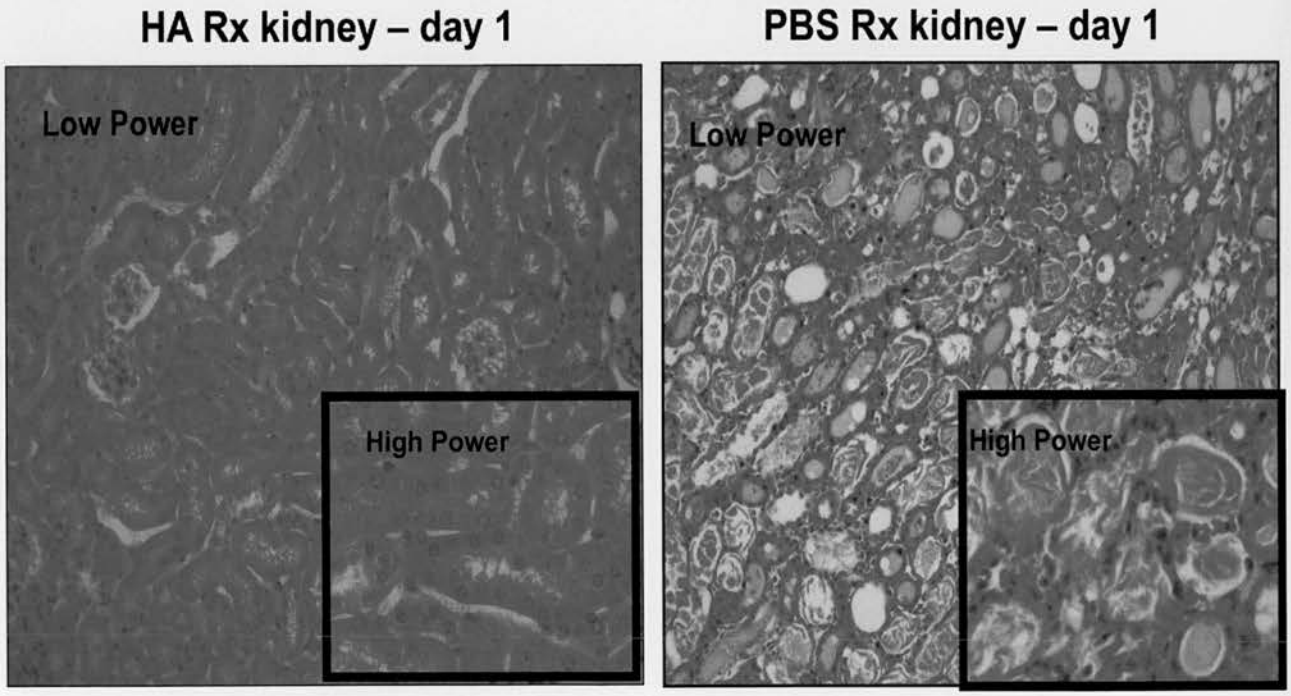


Fig 3.5: Comparison of PBS and HA treated kidneys following IRI. Extensive ATN is observed in the PBS pre-treated kidney, with marked loss of tubular epithelial cells. There is cast material and debris within many of the tubules and microscopic evidence of haemorrhage within the tissue. By contrast, the HA pre-treated kidney has preserved tubules with viable tubular epithelial cells (low power x40, high-power x200 magnification)

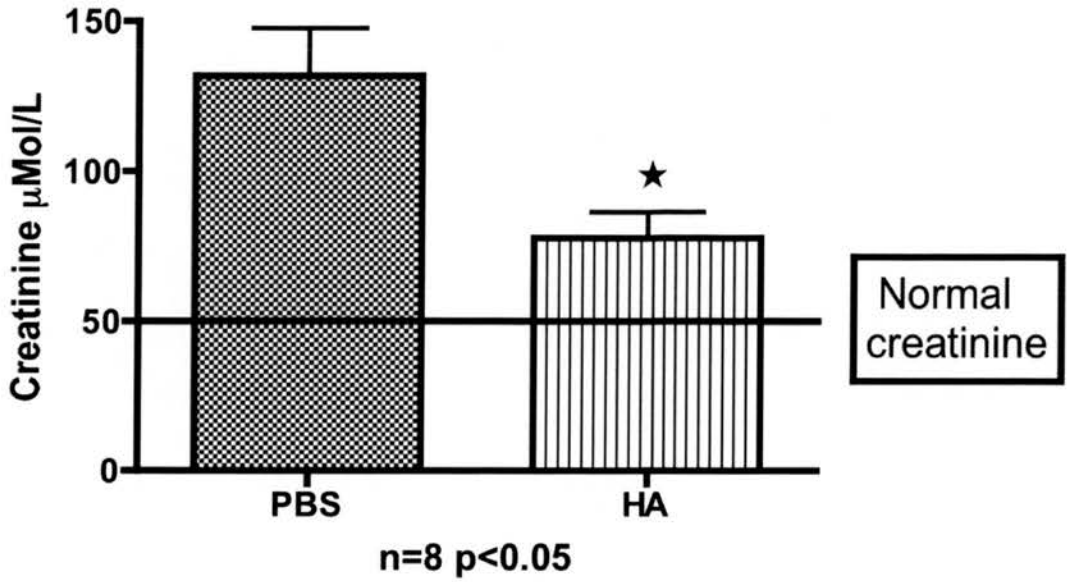


Fig 3.6: Pre-treatment with HA significantly protects renal function against IRI compared with PBS pre-treated control animals (n=8, $p<0.05$)

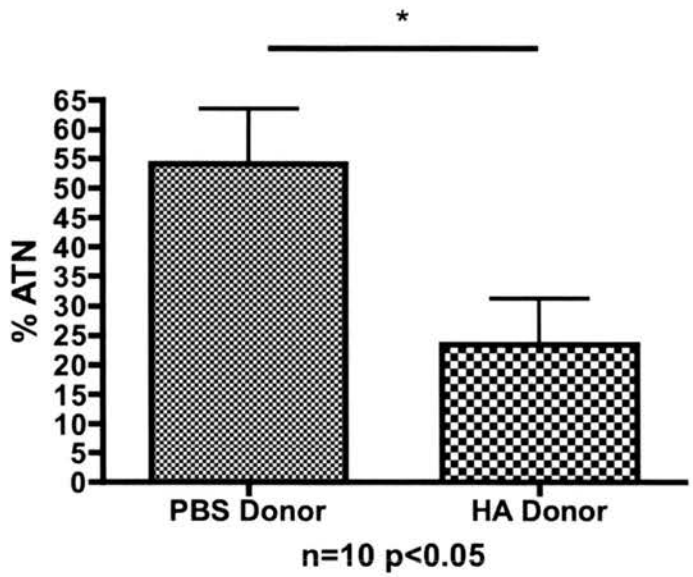


Fig 3.7. Renal Isograft transplantation: Pre-treatment of the organ donor with HA results in significantly lower ATN scores compared with PBS pre-treated control animals following transplantation (n=10, $p<0.05$)

Pre-treatment of the organ recipient with Heme arginate offers relative protection of renal structure in murine renal isograft transplantation

A clear trend toward a reduction in ATN scores was seen in HA treated recipient animals when compared with their PBS treated counterparts although these differences did not reach statistical significance in this series of experiments. This is largely due to the lower ATN scores in the PBS control recipient treated animals compared with those in the PBS donor treated animals. Reasons for this difference are unclear. The result may reach statistical significance if additional transplant experiments are performed. (Fig 3.8. 29.1 ± 8.5% vs. 14.2 ± 4.5% ATN. PBS vs. HA; n=10, p=0.21).

Pre-treatment with Heme arginate offers relative preservation of the renal microvasculature following native kidney IRI

Given the marked differences in levels of acute tubular necrosis and renal function, the integrity of the vascular network was assessed using the endothelial cell marker CD31. Representative images of CD31 staining within the outer medulla prior to injury compared with images taken following IRI in HA and PBS treated animals are seen in Fig 3.9. There was loss of CD31 staining in the outer medulla of PBS treated animals 24 hours after IRI, although the differences did not reach statistical significance (Fig 3.10. 15.2 ± 3.5 vs. 8.7 ± 1.3; %OSOM CD31+ve; PBS day 0 vs. PBS day 1; n=8, p=0.08). CD31 positivity was relatively preserved in HA pre treated animals after injury by comparison, although this result was not statistically significant (Fig 3.10. 11.8 ± 2.0 vs. 13.1 ± 2.0; %OSOM CD31+ve; HA day 0 vs. HA day 1; n=8, p>0.05).

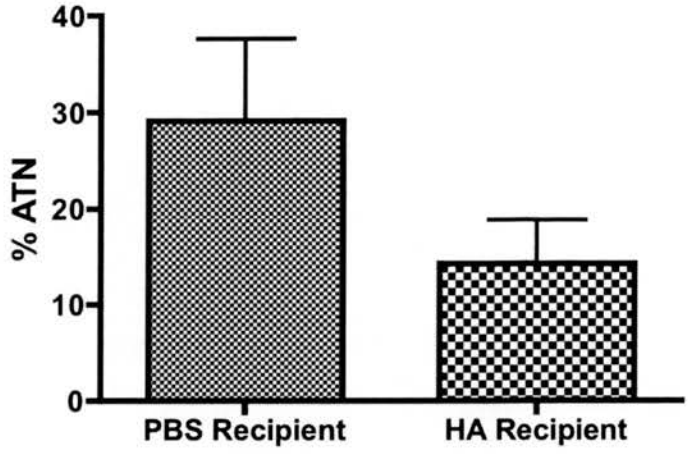
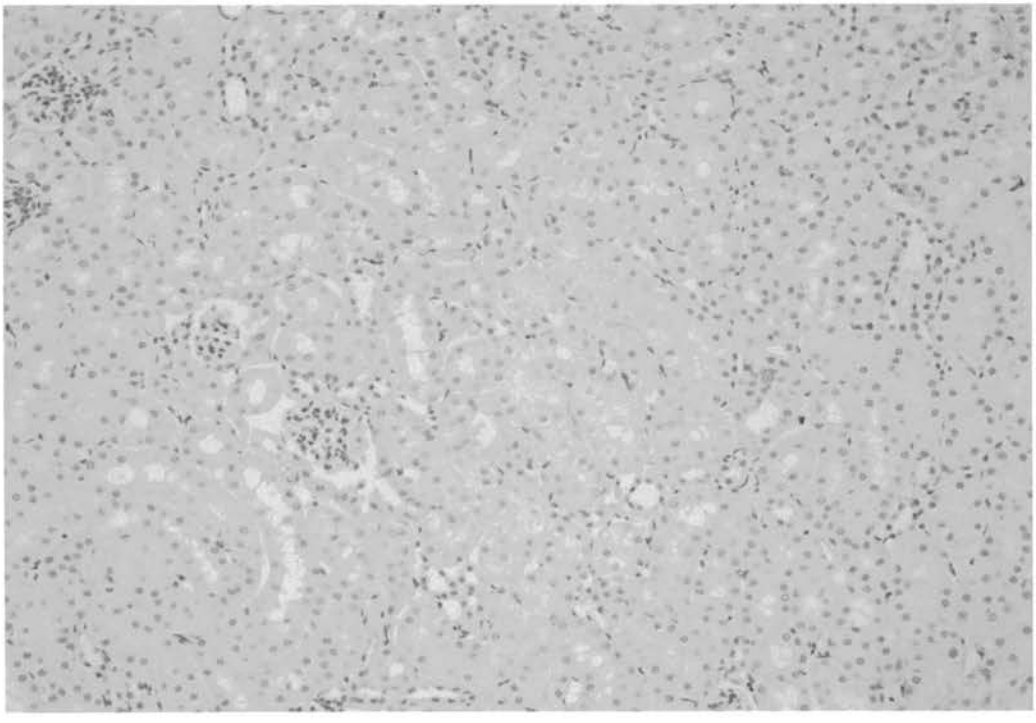
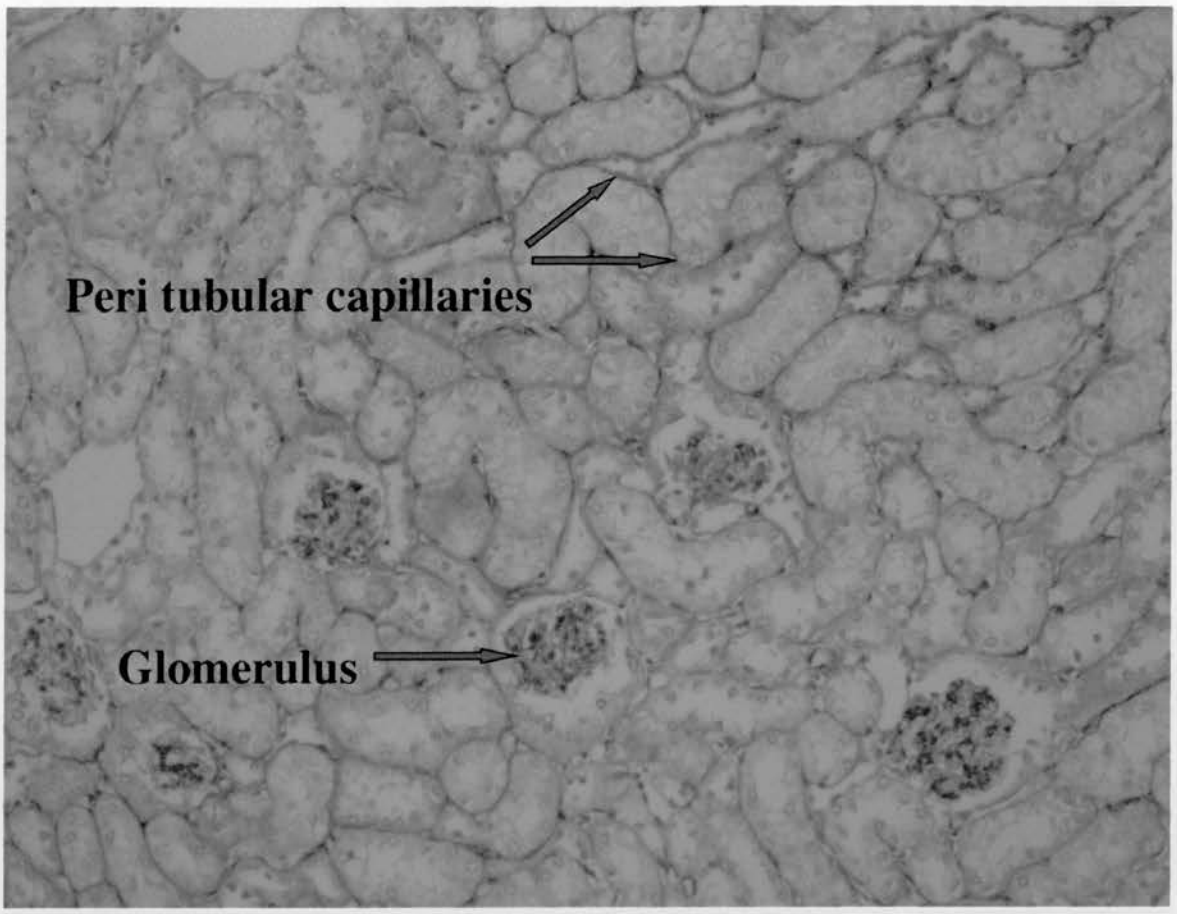


Fig 3.8 Renal isograft transplantation: Pre-treatment of the organ recipient with HA results in a trend toward lower ATN scores when compared to PBS treated control animals. This does not reach statistical significance (n=10, p=0.21)

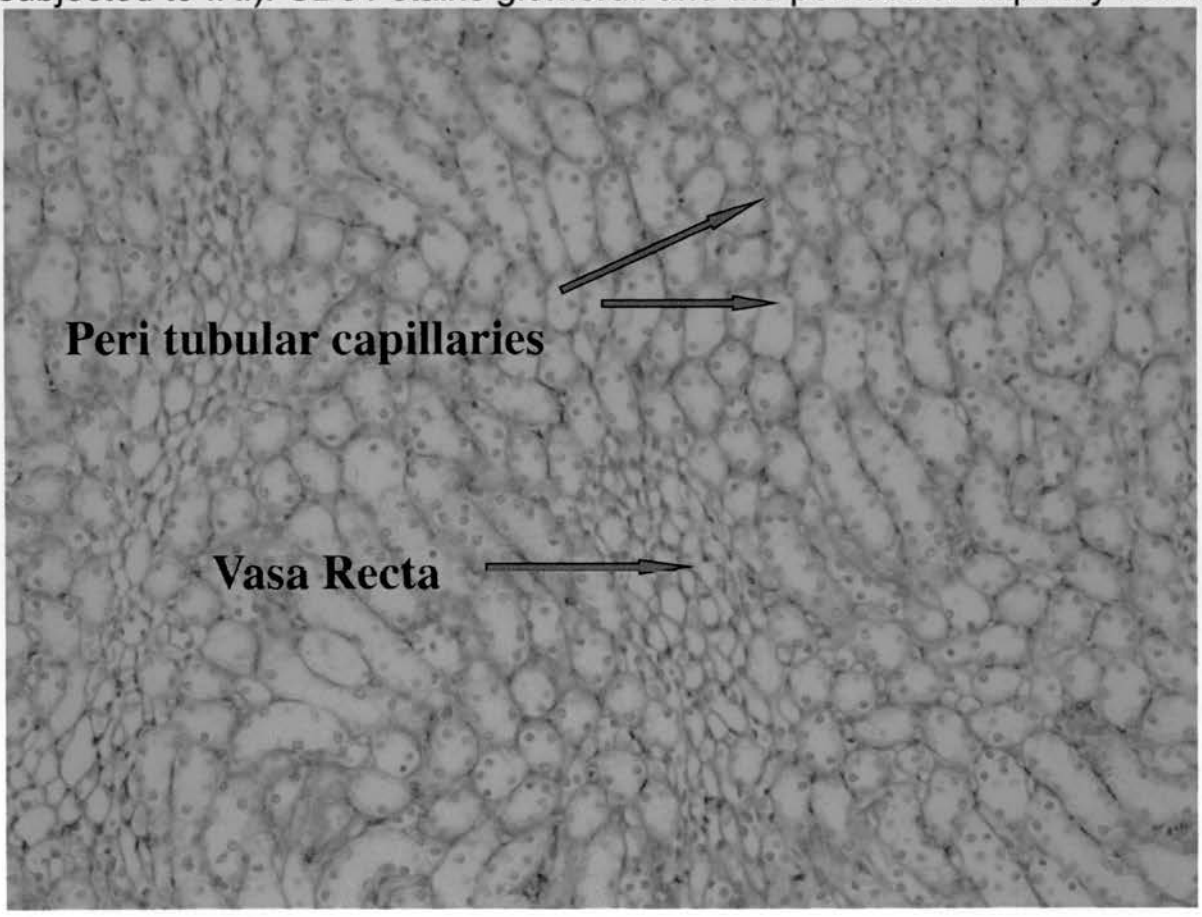
Fig 3.9



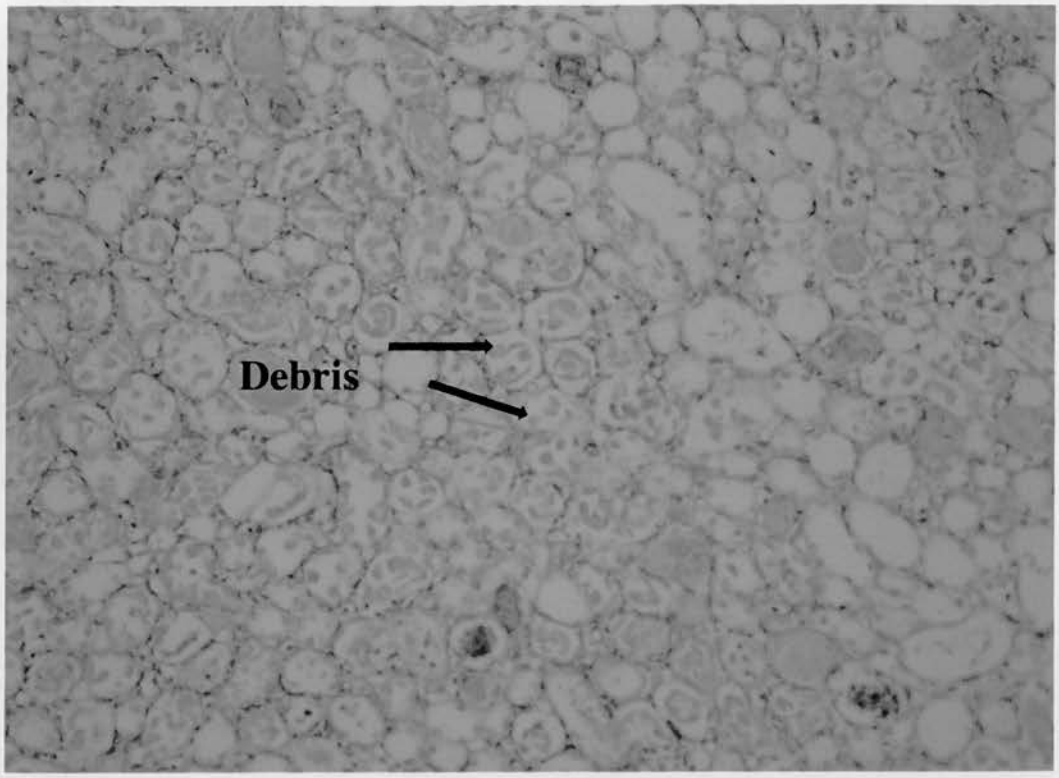
Isotype control. Section of renal cortex incubated with rat igG1, the isotype control for CD31. No positive staining is observed.



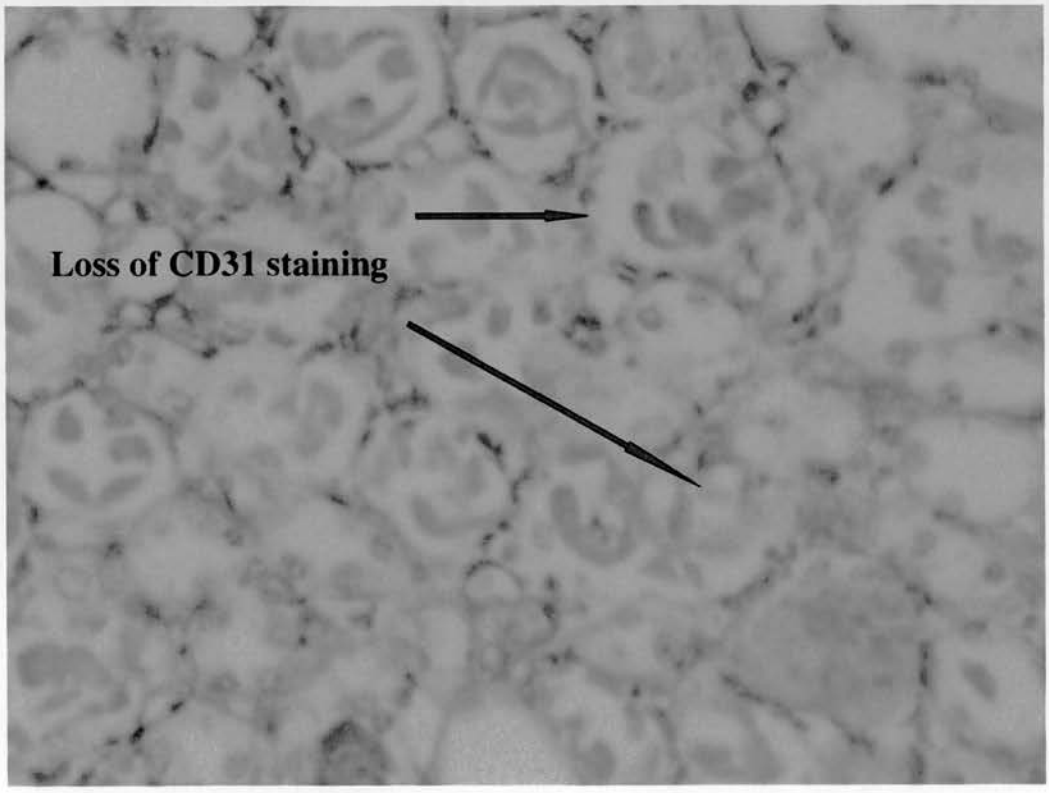
CD31 renal cortex. x20 image from contra-lateral control kidney (day 0 – not subjected to IRI). CD31 stains glomeruli and the peritubular capillary network.



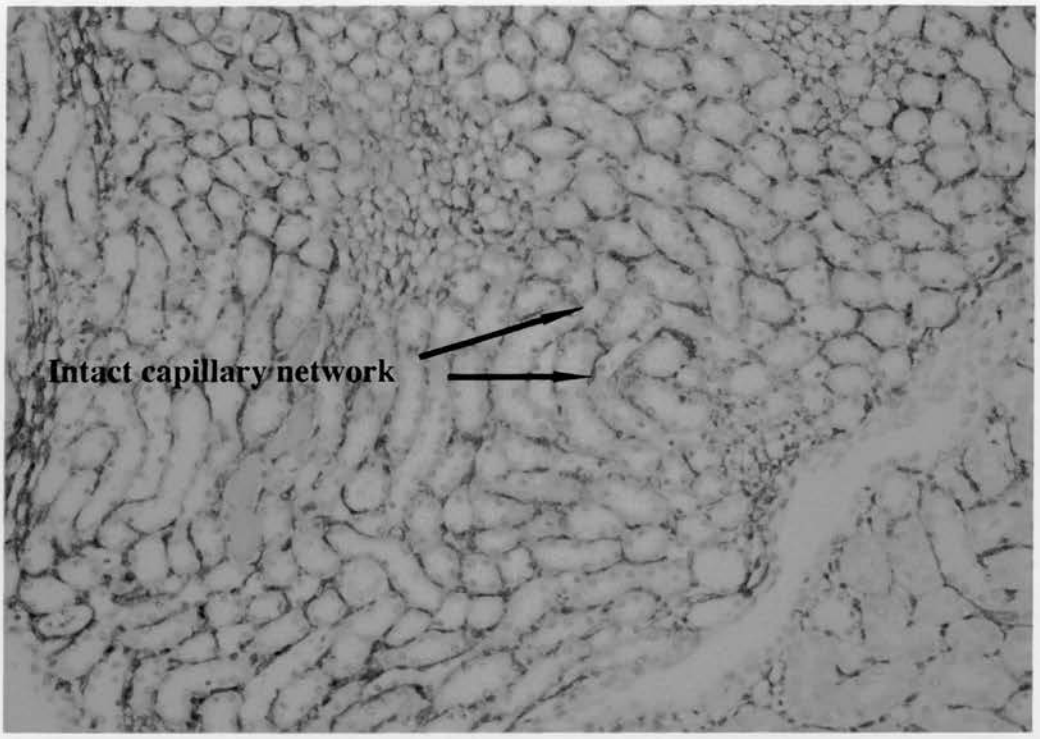
CD31 Outer medulla. x20 image from day 0 kidney. CD31 delineates the intact peritubular capillary network and vasa recta.



CD31 Outer medulla, day 1. PBS pre-treatment: X20 image from the outer medulla. There is extensive ATN with necrotic tubular debris



CD31 Outer medulla, day 1. PBS pre-treatment: X200 image from the same slide. The peri-tubular capillary network is clearly disrupted



CD31 Outer medulla, day 1. HA pre-treatment: By contrast, in this X20 image from the outer medulla of an HA pre-treated animal, there is preservation of the peri-tubular capillary network.

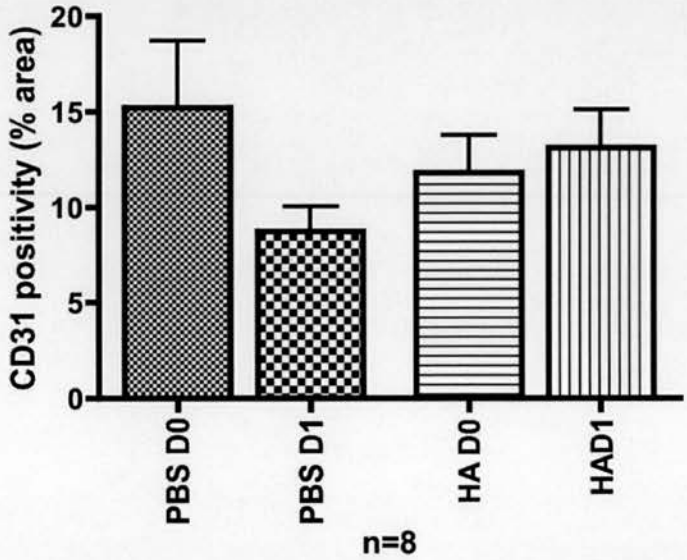


Fig 3.10. Native kidney IRI: Counts of CD31 positivity by percentage area taken from the outer medulla in PBS and HA treated animals prior to (day 0) and following IRI (day 1). There is a clear trend toward loss of CD31 staining in PBS pre-treated animals, although this does not reach statistical significance (n=8, p=0.096). By contrast, CD31 positivity remains constant in the HA treated animals following injury, implying preservation of the peritubular capillary network.

Pre-treatment of the organ donor with HA does not protect the renal microvasculature following renal isograft transplantation

There was a significant loss of CD31 immunostaining in the outer medulla of recipients whose organ donor was treated with either PBS or HA when compared with pre-transplantation levels from the donors contralateral kiney (day 1). These results suggest that HA treatment did not preserve the integrity of the microvasculature in the HA treatment group, despite the significantly lower ATN scores observed following HA treatment (Fig 3.11. 25.2 ± 3.7 vs. 12.3 ± 3.7; %OSOM CD31+ve; PBS D1 vs. PBS D2; n=10, $p<0.05$. 24.8 ± 2.4 vs. 10.3 ± 2.7; %OSOM CD31+ve; HA D1 vs. HA D2; n=10, $p<0.05$).

Recipient animals pre-treated with either HA or PBS have similar CD 31 counts before and after renal isograft transplantation

There were no statistically significant differences in the levels of CD31 immunostaining in the outer medullas of either PBS or HA treated recipient animals when compared to the levels in the day 1 nephrectomy specimen from the recipient animal. This result contrasts with the findings in donor animals in that it suggests that transplantation per se does not result in microvascular injury. Potential explanations are discussed below (Fig 3.12. 17.4 ± 2.4 vs. 17.7 ± 2.3; %OSOM CD31+ve; PBS D1 vs. PBS D2; n=10, $p>0.05$. 22.3 ± 2.7 vs. 21.6 ± 3.1; HA D1 vs. HA D2; n=10, $p>0.05$)

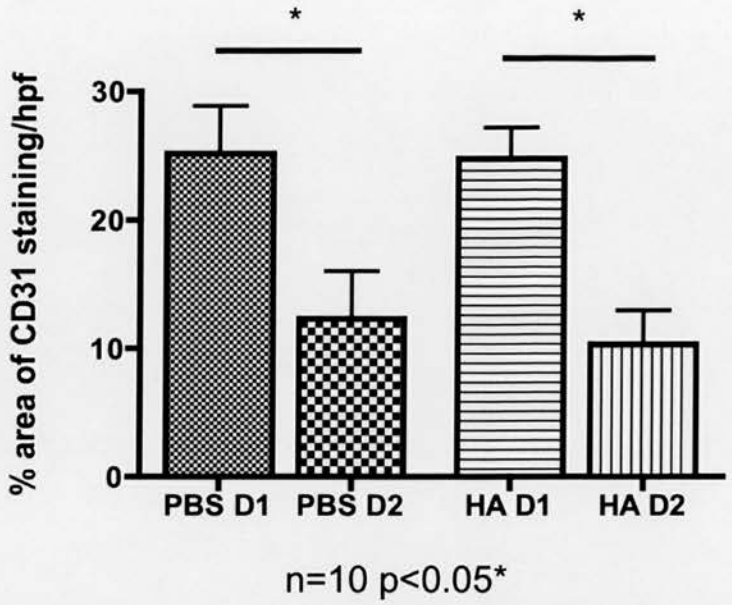


Fig 3.11. Donor pre-treated renal isograft transplantation: Significant reductions in CD31 positivity were seen in both PBS and HA treated donor animals following transplantation compared with non-transplanted contralateral control kidneys. This implies that microvascular injury following transplantation was not modified by HA pre-treatment (n=10, $p<0.05$ for both PBS and HA pre-treatment).

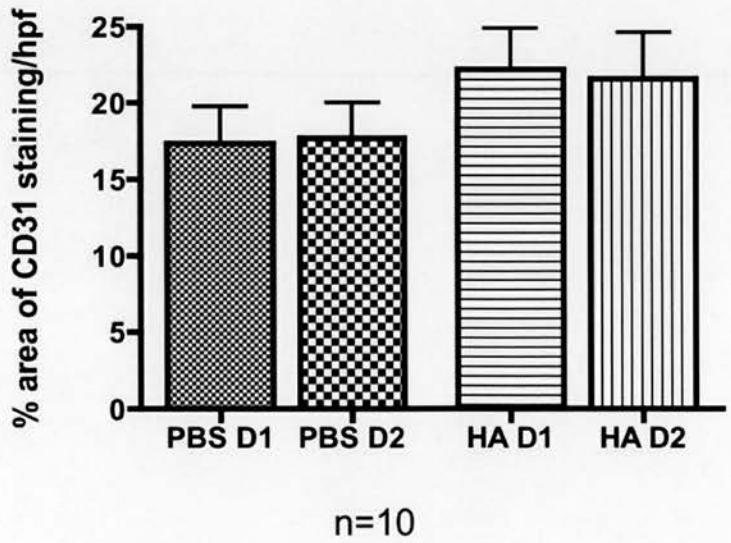


Fig 3.12. Recipient pre-treated renal isograft transplantation: No significant differences were seen in CD31 counts taken from Pre-treated recipient animals following renal isograft transplantation when compared with contralateral control kidneys (day 1) (n=1-, $p>0.05$ for both PBS and HA pre-treatment).

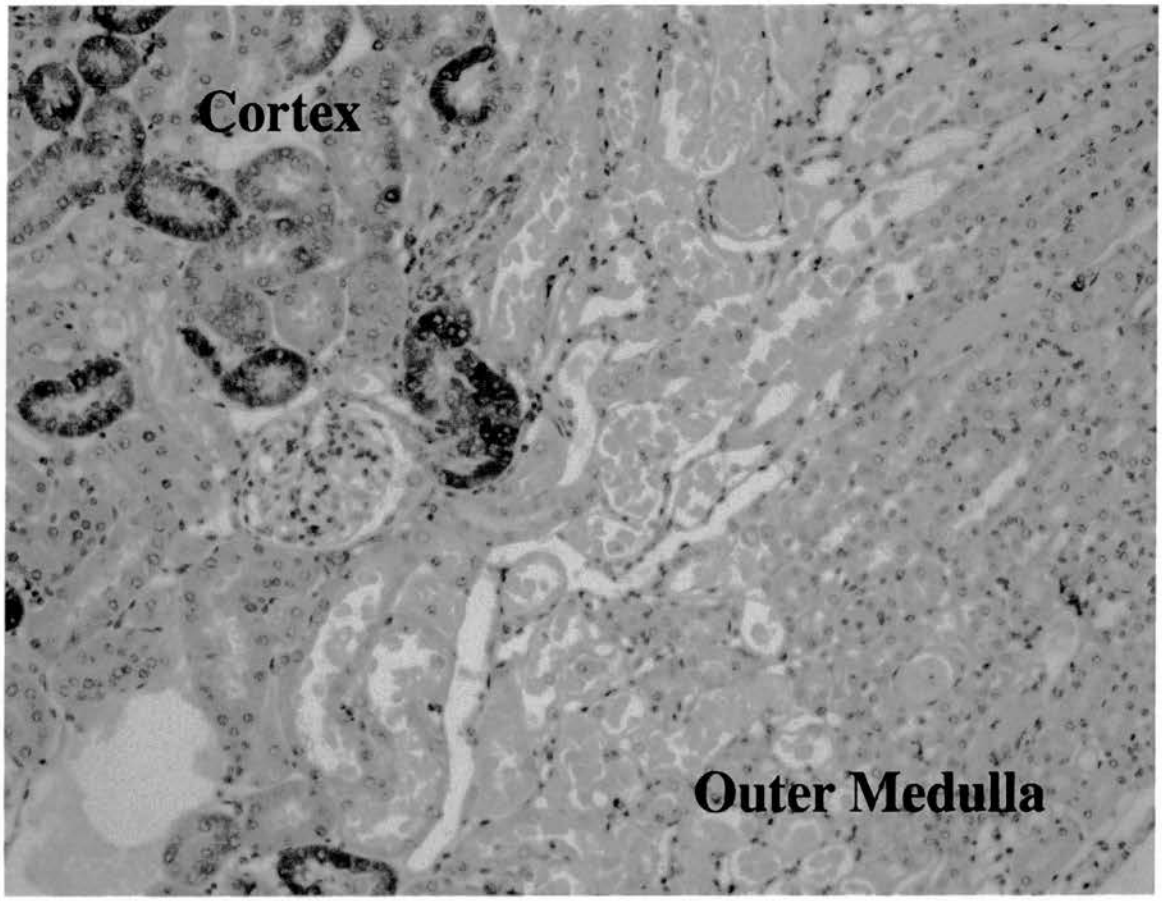
HO-1 positive macrophages are present in the outer stripe of the medulla of HA treated animals before and after native kidney IRI

Immunohistochemistry showed continued expression of HO-1 by the tubules of the renal cortex at the conclusion of the experiment on "day 1". As previously noted in chapter 2, there was minimal expression of HO-1 in the tubules of the outer stripe of the medulla of HA pre-treated mice. This is the area maximally affected by IRI. HO-1 positive interstitial cells were detected in this region prior to injury in the HA-treated group. Dual immunofluorescence confirmed that many of these cells were positive for both the tissue macrophage marker CD68 and HO-1, implying that they are HO-1 positive macrophages (Fig 3.13). The numbers of these cells tended to increase following surgery. In PBS pre-treated animals no such cells were seen before IRI; however multiple HO-1 positive cells were noted 24 hours after surgery even in the absence of HA administration. (Fig 3.14. Cell counts/hpf; 0 ± 0 vs. 2.0 ± 0.65 PBS D0 vs. PBS D1. 2.6 ± 1.17 vs. 3.8 ± 1.1 HA D0 vs. HA D1; n=7-10, $p<0.05$).

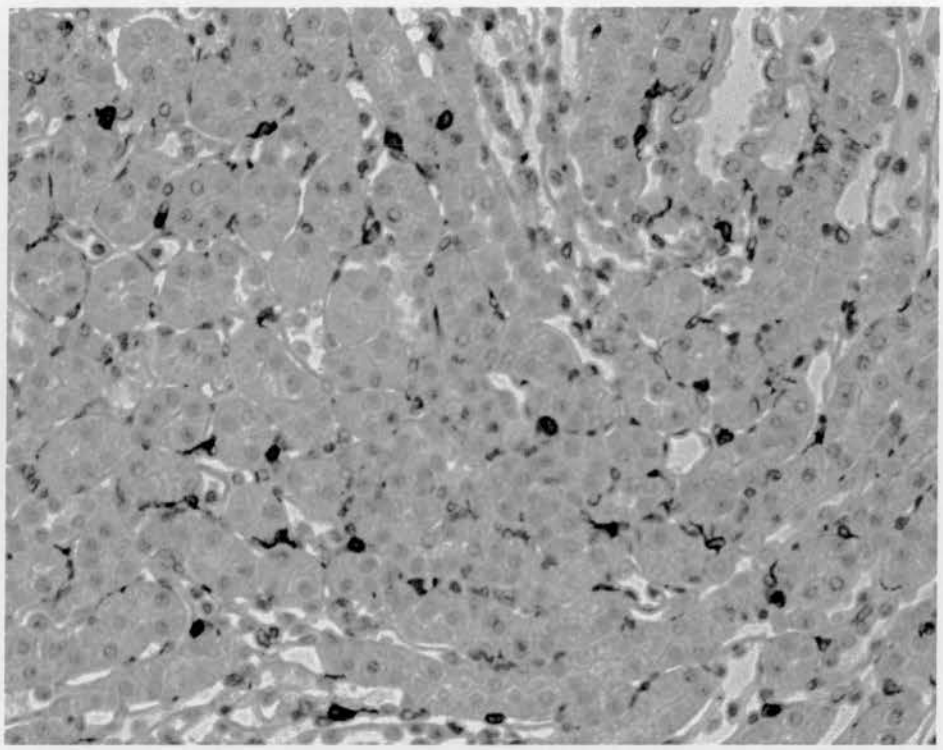
HO-1 positive macrophages are present in the outer medulla of HA treated donor animals before and after renal transplantation

A similar pattern to that seen in native kidney IRI was observed before and after transplant surgery. HO-1 positive cells were present in the outer medulla of donor animals treated with HA, and the numbers of these cells were maintained in the post transplant kidney on day 2. In the PBS control animals HO-1 positive cells were largely absent in the PBS treated animals on day 1, yet are seen at the conclusion of the experiment on day 2 even in the absence of HA treatment (Fig 3.15. Cell counts/hpf; 0.06 ± 0.04 PBS D1 vs. PBS D2. 6.4 ± 2.4 vs. 12.4 ± 3.3 HA D1 vs. HA D2; n=10, $p<0.05$).

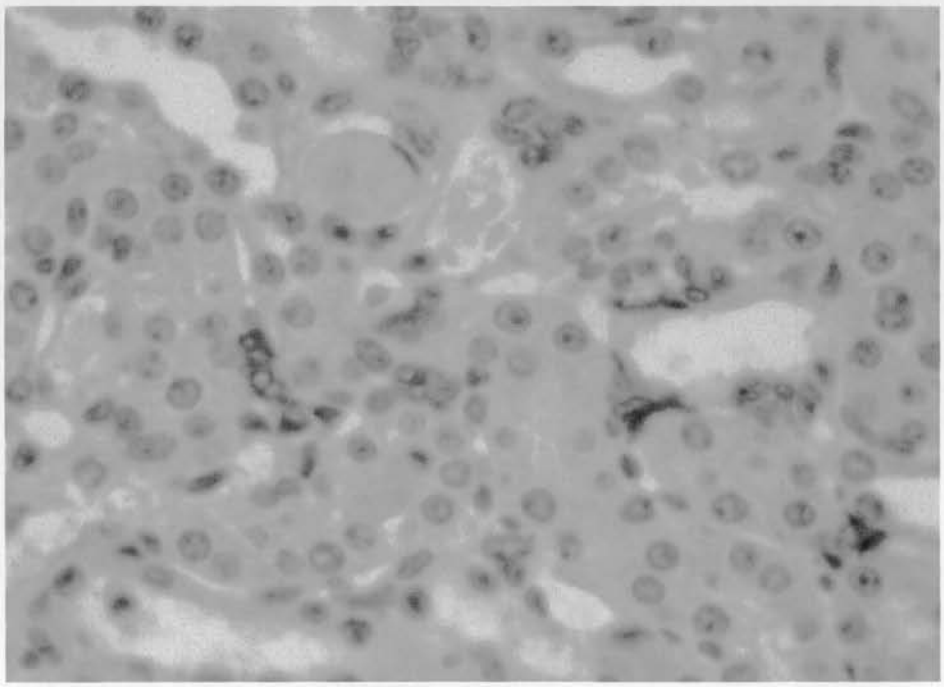
Fig 3.13



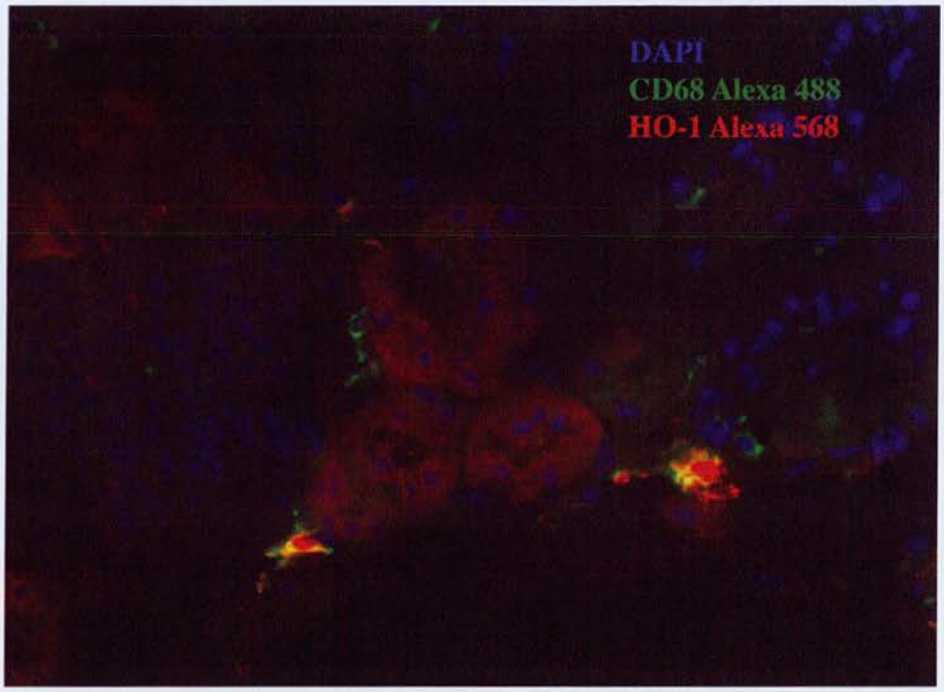
X40 image, HA pre-treated animal, day 1 post IRI: Tubules within the renal cortex continue to express HO-1 at the conclusion of the experiment



Outer medulla of HA pre-treated mouse, day 1. X100 image: HO-1 positive interstitial cells are identified in association with viable tubules. Note that the tubules themselves do not express HO-1



Outer medulla of HA pre-treated mouse, day 1. X200 image. HO-1 positive interstitial cells are noted in association with viable, HO-1 negative tubules.



Dual Immunohistochemistry confirms the presence of HO-1 expressing macrophages: In this image from the outer medulla of a HA pre-treated animal subjected to IRI. CD68 is highlighted in green (Alexa 488), HO-1 is highlighted in red (Alexa 568). Dual positive cells appear as yellow.

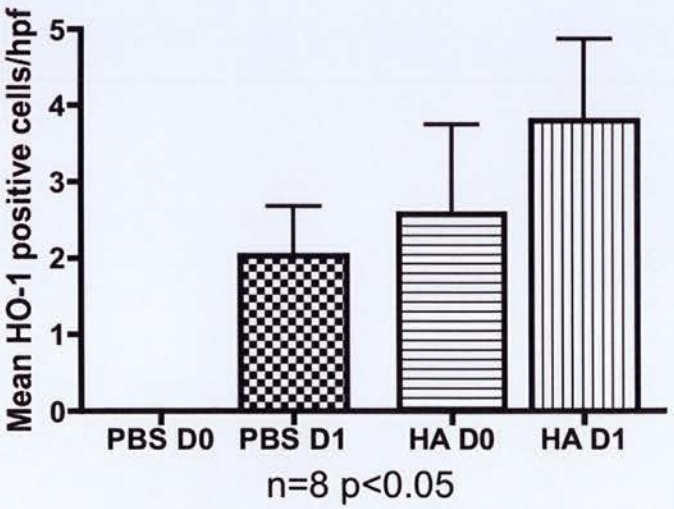


Fig 3.14. Counts of HO-1 positive interstitial cells before and after renal IRI: No HO-1 positive cells were identified prior to IRI in PBS treated animals (day 0). However, even in the absence of HA treatment, HO-1 positive cells were identified after injury (day1). In HA treated animals a population of HO-1 positive cells were present prior to IRI (day 0), their numbers tended to increase after IRI surgery (n=8, $p<0.05$)

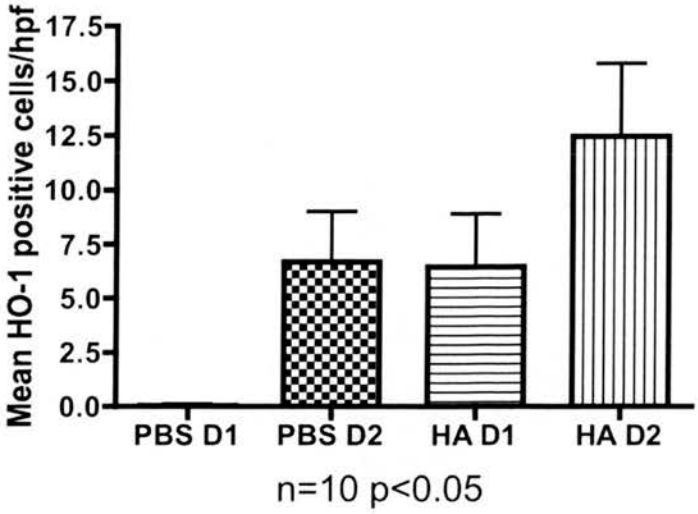


Fig 3.15. Counts of HO-1 positive interstitial cells in the outer medulla following renal isograft transplantation. Donor pre-treatment: Counts of HO-1 positive cells follow a similar pattern to those observed in renal IRI. Increased numbers of HO-1+ cells were present after transplantation in PBS treated donors (day 2) compared with counts from the non-transplanted contralateral kidney (day 1). In HA treated animals a population of HO-1+ cells are present prior to injury (day 1) the numbers of these cells increase after transplantation associated IRI (n=10, p<0.05)

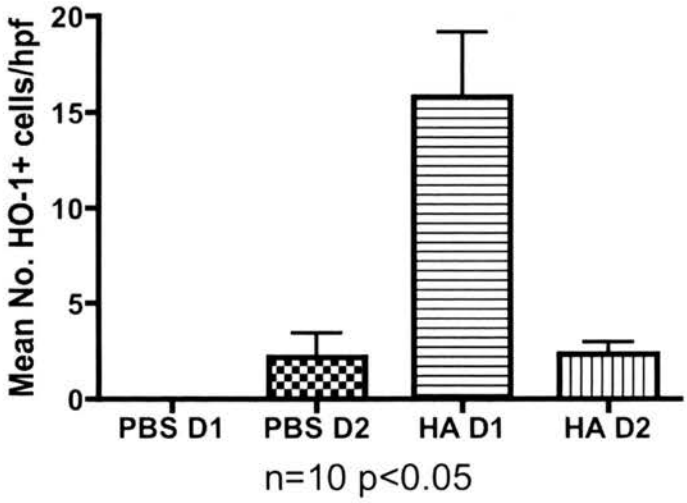


Fig 3.16. Counts of HO-1 positive interstitial cells in the outer medulla following renal isograft transplantation. Recipient pre-treatment: Counts of HO-1 positive cells follow a different pattern in recipient animals pre-treated with HA. The apparent decrease in the numbers of these cells from day 1 to day 2 reflects the fact that the donor kidney, which has not been exposed to HA, has been transplanted into an HA treated animal. The small numbers of HO-1 positive cells present in HA and PBS treated animals on day 2 may represent recruitment of HO-1 positive monocytes from the recipient's circulation, or upregulation of HO-1 by tissue resident macrophages within the transplanted organ itself (n=10, p<0.05)

HO-1 positive macrophages are not present in the outer medulla of the post transplant kidney in HA or PBS treated organ recipients

In recipient animals treated with HA, HO-1 positive cells are present before, injury but their numbers fell significantly following surgery (Fig 3.16, see page 109. Cell counts/hpf; 0 ± 0 vs. 2.2 ± 1.3 PBS D1 vs. PBS D2. 15.8 ± 3.4 vs. 2.3 ± 0.6 HA D1 vs. HA D2; n=10, $p<0.05$). Although the Day 1 kidney has been taken from an animal that has received HA (the recipient), the post transplant kidney (day 2) originated from a donor animal that did not receive HA. Therefore, these HO-1 positive cells have not "disappeared" as such; rather they were never present in the first place. This result implies a lack of recruitment of HO-1 positive macrophages from the circulation 24 hours after surgery. This may in part be due to impaired perfusion of the post-transplant kidney. It may also merely refect the natural history of the chronic inflammatory process in which macrophages typically enter the tissue space at around 48 hours after injury. Analysis of later time points in this model may be of interest

F4/80 counts show no significant differences before or after surgery in either native kidney IRI or renal transplantation

There were no significant differences in the numbers of F4/80 positive cells following IRI (Fig 3.17. Counts of % area/hpf; 10.08 ± 4.6 vs. 2.53 ± 0.75 PBS D0 vs. PBS D1; 5.75 ± 2.84 vs. 4.5 ± 0.9 HA D0 vs. HA D1; n=4-9, $p>0.05$). There were also no significant differences in the numbers of F4/80 positive cells pre/post transplantation in donor pre-treated animals (Fig 3.18. Counts of % area/hpf; 0.64 ± 0.12 vs. 0.9 ± 0.34 PBS D1 vs. PBS D2; 1.46 ± 0.51 vs. 0.81 ± 0.28 HA D1 vs. HA D2; n=10, $p>0.05$). Similarly, there were no significant differences in the numbers of F4/80 positive cells in recipient pre-treated animals following transplantation (Fig 3.19. Counts of % area/hpf; 1.2 ± 0.53 vs. 0.37 ± 0.15 PBS D1 vs. PBS D2; 1.12 ± 0.34 vs.

0.84 ± 0.18 HA D1 vs. HA D2; n=10, p>0.05). These observations are made despite the changes in the numbers of HO-1/CD68 co-positive cells in transplantation and may reflect the fact that HO-1 positive macrophages constitute only certain a proportion of the total number of F4/80 positive macrophages present in the outer medulla. No consistent pattern was noted in the F4/80 staining between the experiments.

An influx of neutrophils was noted in the post ischaemic tissues of native kidney IRI

Neutrophils were not seen in uninjured renal tissue A significant influx of GR1 positive neutrophils was also noted in the OSOM of both the control and HA treated animals following IRI surgery. (Fig 3.20, cell counts/hpf 0.0 ± 0.0 vs. 8.22 ± 4.06 PBS D0 vs. PBS D1; 0.34 ± 0.08 vs. 8.87 ± 2.29 HA D0 vs. HA D1; n=7-9 p<0.05). Neutrophils are associated with acute inflammation and tissue necrosis. Their presence in association with necrotic tubules is a consistent and expected finding.

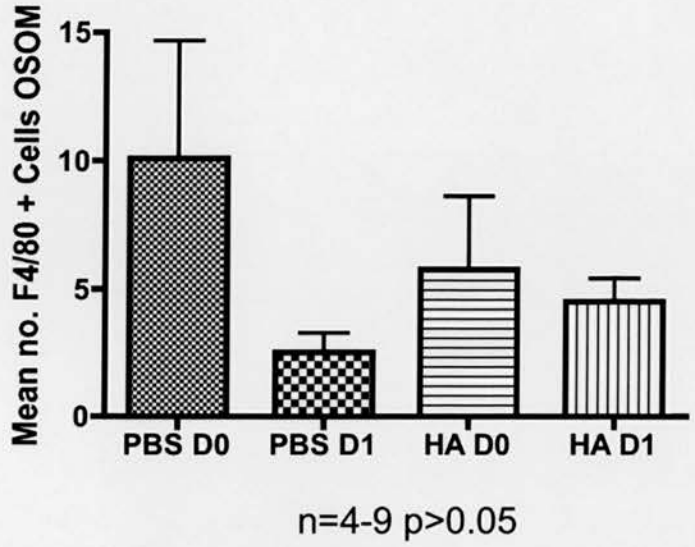


Fig 3.17. Counts of F4/80 positive cells in the outer medulla native kidney IRI: There are no significant differences in the counts of F4/80 positive cells (mean % area/hpf) before or after IRI in HA or PBS treated animals (n=4-9, p>0.05)

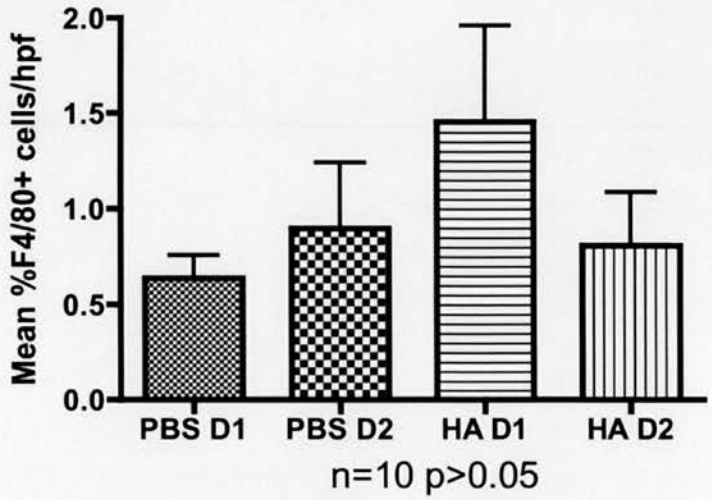


Fig 3.18. Counts of F4/80 positive cells in the outer medulla, donor Rx isograft transplantation: There are no significant differences in the counts of F4/80 positive cells (mean % area/hpf) before or after transplantation associated IRI in donor pre-treated animals (n=10, p>0.05).

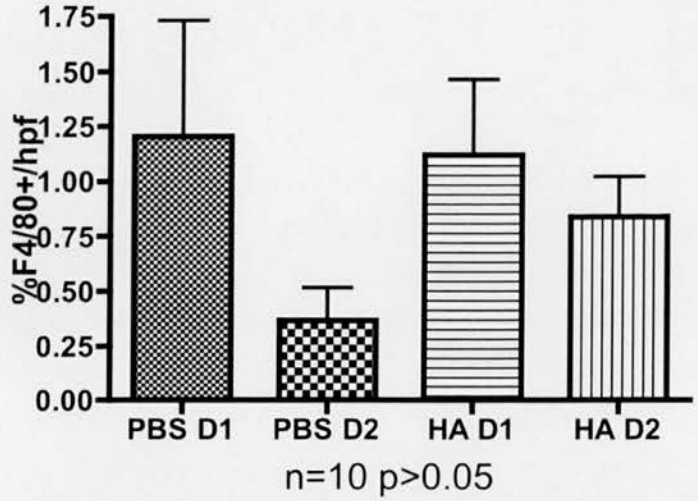


Fig 3.19. Counts of F4/80 positive cells in the outer medulla, recipient Rx isograft transplantation: There are no significant differences in the counts of F4/80 positive cells (mean % area/hpf) before or after transplantation associated IRI in recipient pre-treated animals (n=10, p>0.05).

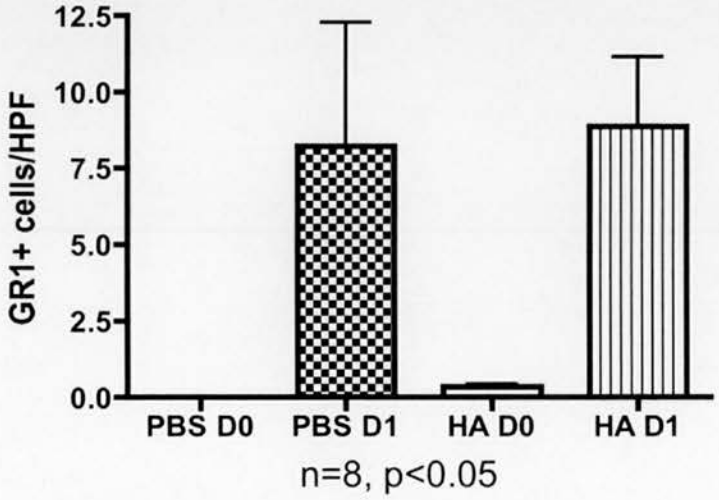


Fig 3.20. Counts of GR1 positive cells in the outer medulla native kidney IRI: A significant increase in the numbers of neutrophils were observed in the outer medulla of both HA and PBS treated animals following IRI (n=8, p<0.05)

Summary

The dataset presented in this chapter demonstrates the following key points:

1. HA protects renal structure and function in a murine model of warm renal IRI.
2. HA mediated protection in IRI is associated with relative preservation of the renal microvasculature.
3. HA protects renal tubules, but not the microvasculature, when administered to donor mice in a model of renal isograft transplantation.
4. HA treatment offers relative protection of renal tubules when administered to recipient animals in renal isograft transplantation.
5. HA mediated protection is associated with HO-1 positive interstitial cells in the outer medulla of both IRI and "donor treated" transplants. HO-1 positive cells were not seen in the outer medulla of "recipient treated" transplants.

Discussion

These experiments utilise established, stable experimental models of renal IRI[50, 67] and renal isograft transplantation[219, 276] which result in relatively consistent levels of ATN. The severity of Injury in IRI is influenced by variation in temperature[277-279], accordingly, uniform experimental conditions were rigorously maintained across all experimental groups with the use of controlled heating blankets and rectal temperature probes.

These data demonstrate that HA treatment prior to surgery protects both renal structure and function in a murine model of native kidney IRI. This work also suggests that treatment of the organ donor animal is protective against transplant associated IRI in a murine model of renal isograft transplantation. A clear trend toward protection following transplantation was also observed in recipient treated animals, although the differences between the treatment and control groups did not reach statistical significance. Changes within the structural integrity of the microvasculature were less consistent. There was a clear trend toward preservation of the peritubular capillary network within native kidney IRI (as measured by CD31 immunostaining). By contrast, protection of renal tubules within HA treated donor animals following transplantation was not associated with preservation of the renal microvasculature. No alterations in the CD31 counts were seen in the recipient treated animals before and after surgery in HA or control groups. HO-1 positive interstitial cells were present within the outer medulla prior to injury in HA treated animals subjected to native kidney IRI. The numbers of these cells were maintained following surgery. A similar pattern was observed in HA treated donor animals. By contrast, no such cells were observed in PBS treated controls prior to injury, however a population of HO-1 positive cells were identified following injury, even in the absence of HA treatment. Whilst HO-1 positive cells were observed in the nephrectomy specimens taken from HA treated recipients on day 1, HO-1 positive interstitial cells were not

observed in the post transplant kidneys of these animals. These findings are discussed below.

HA treatment protects both renal structure and function following native kidney IRI. Previous experience from within our group with this model of renal IRI suggests that preservation of renal function following therapeutic intervention does not always correlate with reduced tubular injury. Ferenbach et al[67] showed that macrophages that were adenovirally transformed to over-express HO-1 protected renal function in mice subjected to IRI, despite there being similar levels of ATN in both treatment and control groups. This latter observation is relevant to one of the central hypotheses to this thesis. It suggests that whilst the survival of tubular cells is clearly important for the preservation of renal function following IRI, alternative factors might also be relevant. As discussed earlier, the effects of IRI upon the renal microvasculature are profound. The effects of platelet aggregation, vasoconstriction and endothelial dysfunction may play a critical role in reducing the perfusion of the remaining tubules following surgery, thereby worsening renal function and potentiating downstream ischaemic damage[194, 280]. Ferenbach et al observed a reduction in platelet aggregation in mice injected with HO-1 over-expressing macrophages, and suggest that CO elaborated as an end product of the reaction catalysed by HO-1 may be responsible for this effect[275]. Therefore, preservation of renal function in these animals may be as a result of enhanced perfusion of the residual tubules.

The in vitro experimental data has demonstrated that HA pre-treatment protects murine endothelial cells against oxygen and carbon dioxide tensions similar to those that are encountered during in vivo IRI[239]. Addition of the specific HO-1 inhibitor ZNPPiX, abrogated this effect, implying that HO-1 was responsible for this protection. The effects of HA treatment upon the protection of the renal microvasculature in vivo were inconsistent. There was a clear trend toward preservation of the renal

microvasculature in HA treated animals subjected to native kidney IRI. It is inferred that HO-1 expression is responsible for this although as was noted in the dose response experiments and in contrast to my in vitro findings, HO-1 expression was not observed in endothelial cells of the peri-tubular capillary network in vivo. This observation is in keeping with the findings of other investigators (Mason JC, unpublished data). In contrast to the findings in native kidney IRI, there was no protection of the microvascular structure by HA treatment in donor treated animals following transplantation. CD31 counts in post-transplant, day 2 kidneys from treated organ donors were significantly reduced in both PBS and HA treatment groups. Results from the recipient treated transplant series suggested that there was no loss of CD31 positivity in either the HA or control groups following surgery. This finding conflicts with the loss of CD31 staining that was observed in native kidney IRI and donor treated transplantation. CD31 is well recognized as a marker of B-lymphocytes and plasma cells[281], platelets and megakaryocytes[282, 283] and has be found by some authors to stain lymphatic vessels[284-286]. It seems unlikely that staining of B-lymphocytes and lymphatics would be a significant source of experimental error. However, It is possible that platelet aggregation, which is commonplace following surgical intervention, may have artificially elevated the CD31 count. This may potentially have given a false impression of microvascular preservation. To avoid this the photoshop settings were carefully adjusted to minimize background "pickup" and an identical immunohistochemical protocol was used to that which was employed in the native kidney IRI and the donor treated transplants. Despite these measures, artefact may still have been an issue. Attempts were made to use alternative endothelial markers such as MECA-32, which is reported to have a high specificity for the endothelium[287]. Unfortunately, this antibody produced very inconsistent staining in both fixed and frozen tissue. The staining technique with CD31 was, however, robust enough to produce meaningful results when used in the native kidney

IRI model and in donor treated renal isografts, which does suggest that the staining protocol and quantification method was valid. Alternatively, these results may mean that that the microvasculature was not significantly injured in either the PBS or HA treated groups in these experiments. The level of ATN was substantially lower than that seen in the donor treated transplant series, which supports this theory. These results as a whole might seem to refute the hypothesis that the maintenance of the microvasculature is critical to tubular survival, however, effects upon the physical structure of blood vessels must be considered within the wider context of the beneficial effects that HO-1 over-expression may have upon a variety of factors that influence renal haemodynamics such as vasodilatation, platelet aggregation and endothelial activation. These indices influence both perfusion and leucocyte infiltration and have been shown to be correlated with beneficial clinical outcomes[66, 199, 200]. The physical integrity of the microvasculature might not entirely reflect all of the potentially important changes within the vascular bed. Probing the relative function of the renal microvasculature would require a multi-modality approach that utilises emerging imaging techniques such as intra-vital microscopy or laser Doppler ultrasound scanning.

A consistent finding within our native kidney model of IRI was the presence of HO-1 positive interstitial macrophages within the outer stripe of the medulla, which is the site of maximal injury following native kidney IRI and transplantation, given its precarious vascular supply. These HO-1 positive cells were observed prior to IRI in HA treated animals and their numbers were maintained following surgery. These cells were also noted following IRI in PBS treated control animals. This may reflect a response to the ischaemic insult itself, which is a recognised mode of HO-1 induction[288]. A similar pattern of results was observed in the donor treated transplant experimental series, in which HA treatment was associated with lower ATN scores. The potential significance of these HO-1 positive cells is highlighted given that the

renal tubules of the outer stripe of the medulla do not express HO-1 in response to HA administration. Previous work from within our group has shown that selective depletion of macrophages in CD11b DTR mice abrogates the protective benefits of HA treatment in renal IRI[50], which implies that HO-1 expressing macrophages may mediate the protective effects of HA. Other authors support this conclusion. HO-1 positive macrophages are reported to mediate some of the protective benefits of statins in a rat model of renal IRI[224] and depletion of HO-1 expressing macrophages is deleterious in hepatic IRI[222]. Furthermore, adenovirally transformed macrophages that over-express HO-1 home to the site of injury upon re-injection and improve renal function in a murine model of renal IRI[67]. Such findings might be explained by the fact that HO-1 over-expression is thought to induce an anti-inflammatory phenotype within macrophages[67, 251, 252]. Interestingly, whilst there was a trend toward protection against ATN in HA treated transplant recipients; the association with the presence of HO-1 positive cells in the outer medulla was less clear-cut. HO-1 positive cells were present in the outer medulla of HA treated recipient animals before injury, but the numbers of these cells were significantly lower after injury. This might suggest an inability to recruit these cells from the circulation of the HA treated recipient animal, which may potentially reflect impaired perfusion of the transplanted organ in the immediate aftermath of surgery. Further mechanistic studies are warranted to clarify the precise role of HO-1 expressing macrophages in this model and to identify additional systemic factors that might be responsible for the protective effects of HA treatment. There was a clear trend toward structural protection in HA treated recipient animals that did not reach statistical significance. The finding that recipient HO-1 over-expression is protective is in IRI is not without precedent. Araujo et al[171] reported that systemic upregulation of HO-1 within recipient animals, rather than local upregulation of HO-1 within the transplanted organ itself improved cardiac allograft outcomes in transgenic mice. These authors attributed the differences they

observed to increased production of carbon monoxide (CO) within the HO-1 over-expressing recipient animals. Indeed, exogenous administration of low dose CO has been shown to protect rat lung transplants from IRI via activation of the p38 MAP kinase pathway[289] and to inhibit progressive chronic allograft nephropathy and restore renal allograft function[290]. The finding that HO-1 positive cells were not present within the outer medulla of HA treated recipient animals suggests that other factors may be involved in mediating the protection by HA. Increased CO, produced as a consequence of HO-1 activity, may potentially play a role in the mechanism of protection observed in HA-treated recipient animals, in particular its effects upon platelet deposition may be relevant[67]. However, preliminary attempts to measure CO levels in the blood of HA treated mice proved unsuccessful. This may be related to the sensitivity of the analytical equipment available for use.

Future work

The significance of these experiments is that they suggest that HA, a drug that is already clinically licensed and in regular use (albeit for an alternative indication), might afford protection in when administered to donor animals in models of transplantation associated IRI. The work also suggests a clear trend toward renal tubular protection in recipient treated animals. This result may have reached statistical significance had a larger number of experiments been performed. There are a number of key limitations of this experimental data that are acknowledged. These issues could be pursued through additional studies. The data as presented does not definitively show that HO-1 itself is responsible for HA mediated protection and therefore repeating this series of experiments with HO-1 "knockout mice" would be an important experiment to clarify the role of HO-1 in HA mediated protection *in vivo*. Furthermore this work does not provide an insight into the fundamental mechanisms underlying HA mediated protection. I have suggested that the protection may be due to a modifying action of the drug upon the microvasculature, the renal tubules, HO-1 positive macrophages or a combination of effects upon a variety of these and other systems, however the results of my experiments are rather contradictory in this regard and I cannot claim to draw any definitive conclusions from my data. My experiments show no clear-cut association between CD31 counts and ATN score across the native kidney IRI and isograft transplantation experiments. The microvasculature does appear relatively preserved following HA administration in native kidney IRI (where HA protected against ATN) and also in HA treated isograft recipients (where there was a trend toward structural protection by HA that fell short of statistical significance). However, HA treated organ donors showed no such preservation of their microvasculature despite being protected against ATN. Furthermore there was no consistent pattern in the counts of HO-1 positive macrophages in the outer medulla when native kidney IRI was compared to donor and recipient treated isograft

transplantation. The numbers of these cells remained relatively consistent both before and after surgery in animals that received HA prior to both native kidney IRI and in HA treated organ donors. The presence of these cells appeared to be associated with significant protection against ATN in both cases. By contrast, very few HO-1 positive macrophages were identified in the post transplant kidneys of organ recipients that received HA pre-treatment prior to surgery. Despite the lack of statistical significance in the ATN scores in this group when compared to their PBS controls, there did appear to be a clear trend toward structural protection in these animals. These inconsistencies suggest that there may be multiple mechanisms involved in mediating the protection against ATN by HA that may include factors other than those mentioned here. There may also be different biological mechanisms involved in systemic treatment when compared to treatment of the organ donor alone. Clarifying the underlying mechanism would be of interest not only from a scientific perspective, but may also be of clinical relevance in terms of how this drug may potentially be administered to transplant patients in the future.

HO-1 positive macrophages

Whilst experiences from within our laboratory group indicate that HO-1 positive macrophages mediate the protective benefits of HA treatment within murine renal IRI[50], we did not observe HO-1 positive cells within the outer stripe of the medulla of HA treated recipient animals, although their ATN scores were lower than PBS controls. The precise origin of these HO-1 positive macrophages *following* surgical intervention has also not been definitively determined in either native kidney IRI or organ donor treated transplants. It is therefore unclear whether they represent tissue resident macrophages in which upregulation of HO-1 occurs in response to ischaemic stress, or whether there is recruitment of HO-1 positive monocytes from the animal's circulation. The capacity to physically dissociate the organ systems, in the context of

transplantation affords an opportunity to examine this. The use of transgenic, MacGreen mice in which macrophages are fluorescent[291] as either organ donors or recipients in combination with non-transgenic animals (as either donor or recipient) might clarify this matter. The lack of HO-1 positive macrophages within the OSOM of recipient animals suggests that recruitment from the circulation may not occur following transplantation at the time point we studied (24 hours post surgery). It may be of value to investigate later time points to see whether such cells are subsequently recruited to this site. Repetition of these experiments using transgenic CD11b DTR mice may establish whether HO-1 positive macrophages mediate the beneficial effects of HA treatment within our transplant model. CD11b-DTR mice (FVB/nj strain) are transgenic for the human diphtheria toxin receptor gene under the control of the CD11b promoter. Administration of a minute dose of diphtheria toxin results in rapid ablation of circulating monocytes and resident/infiltrating renal macrophages. Such mice could be utilised as either organ donors or recipients. Abbrogation of a protective effect against ATN in mice that were administered HA and diptheria toxin might imply that macrophages are necessary to mediate the protective of HA *in vivo*.

The microvasculature

There are inconsistencies in the pattern of CD31 expression across the series of i*n vivo* experiments making it difficult to draw definitive conclusions. As indicated earlier in the discussion, CD31 expression may provide a poor reflection of the range of changes occurring within the microvasculature and the endothelium following transplantation or IRI and a multimodality approach that utilises emerging imaging techniques such as intravital microscopy or infra-red imaging may potentially yield additionally more relevant data including a real time assessment and quantification of renal perfusion following surgical intervention[66, 292]. Staining for CD31 was performed in the case of both native kidney IRI and transplantation upon kidneys that

were retrieved 24 hours following surgical intervention. As such, the values obtained may merely reflect the structural integrity of the whole tissue at this time point. Clearly, our intention was to draw some mechanistic inferences from the relationship between tubular preservation and microvascular integrity. It may be of interest to study the integrity of the microvasculature and tubular injury at earlier time points i.e. immediately after surgery and for example 2, 4,6 and 8 hours after surgery. From this it may be possible to infer a relationship between microvascular disruption and subsequent tubular loss, which might suggest a cause and effect relationship. It would be intriguing to note whether HA treatment preserves the structure of the peri-tubular capillary network at such earlier time points.

Molecular mediators

The breakdown products of the reaction catalysed by HO-1 are thought to mediate the protective benefits attributed to the enzyme[293]. It may therefore be of value to measure CO levels within mice before and after HA administration. Unfortunately my attempts to demonstrate a difference the levels found in HA and PBS treated animals were unsuccessful. I suspect this may reflect a lack of sensitivity on the part of the laboratory equipment used. Other authors have however measured increased carboxyhaemoglobin levels in rats in association with HO-1 induction by hemin[294]. Repition of these transplants with substitution of the isolated end products of the reaction that HO-1 catalyses may also clarify mechanism underlying the protection we observe. Carbon monoxide releasing molecules are commercially available and have been shown to confer protective benefits in renal IRI[248] and as mentioned earlier, exogenous administration of CO has been shown to be beneficial in animal models of lung[289] and renal transplantation[290]. It is likely however that the other breakdown products (biliverdin which is converted to bilirubin and free iron via its depletion and upregulation of ferrritin) may be involved to in some capacity by modulating a variety

of additional factors[167]. Indeed the haemoxygenase system is often thought to have evolved as defense mechanism due to the pluripotential protective effects against cellular stress that its breakdown products exert[172].

Nitric Oxide (NO)

NO is known to exert a variety of biological effects that may be potentially protective against IRI which include vasodilatation[295], inhibition of endothelin-1 production[124, 190], prevention of leucocyte adherence to endothelium[296] and inhibition of platelet aggregation[190]. It is possible that increased NO activity may play a role protection against IRI following HA administration. The L-arginine, to which haem is bound in HA, is a substrate for nitric oxide synthase and may result in the formation of nitric oxide (NO)[297]. Definitive demonstration that HO-1 per se, rather than NO or another intermediary, is responsible for the protective benefits of HA administration could be determined by showing a loss of protection associated with HA treatment in transgenic HO-1 knockout mice following organ transplantation or IRI.

Other future work

From a clinical perspective it may be of obvious interest to evaluate whether a combination of both donor and recipient treatment with HA might provide any additional protection from transplant associated IRI. However, treatment of brain-stem dead organ donors may raise practical and ethical issues[185]. The transplantation experiments presented in this thesis have also been performed entirely within an isograft system. This allows for assessment of the effects of HA treatment upon IRI in isolation, but does not fully simulate the complex interplay of immune factors that are encountered within allograft transplants. It may be of interest to repeat these experiments using mice of different strains to simulate allograft transplantation and assess the effects of the drug upon acute and chronic rejection. The current work is

also based largely upon the contention that the initial severity of IRI is associated with delayed graft function[21, 22] which is a negative prognostic factor for long-term graft survival[21, 23, 72]. A single time point of 24 hours following organ transplantation and native kidney IRI was selected. It would be interesting to assess the effect of HA pre-treatment upon fibrosis, tubular injury and potentially renal function, in transplanted organs at longer time-points after surgery. This may provide a more realistic simulation of the potential benefit of the drug in clinical practice i.e. in reality its main potential benefit would be to improve the long-term outcome after solid organ transplantation. However, a remaining viable kidney in the "non-functional" model described renders measures of serum creatinine non comparable. To date, attempts to establish a stable, functional model of murine renal transplantation within our laboratory group have unfortunately proven unsuccessful.

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Appendix

The Amelioration of Transplant Associated Injury via Hemeoxygenase-1
Oral presentation at the Medawar Medal session, British Transplantation Society Annual Congress. Kensington, London.
March 2010

The Amelioration of Transplant Associated Injury via Hemeoxygenase-1
Oral presentation at the Chiene medal session. School of Surgery Day.
Royal College of Surgeons of Edinburgh. Awarded 2nd place.
November 2009

The Amelioration of Transplant Associated Injury via Hemeoxygenase-1
Oral presentation at the Medawar Medal session, British Transplantation Society Annual Congress. Liverpool.
April 2009

The Amelioration of Transplant Associated Injury via Hemeoxygenase-1
Oral presentation at the Chiene medal session. School of Surgery Day.
Royal College of Surgeons of Edinburgh. Awarded 2nd place.
December 2008

The Amelioration of Transplant Associated Injury via Hemeoxygenase-1
Oral presentation at a Research in Progress seminar.
Centre for Inflammation Research. University of Edinburgh.
October 2008

The Amelioration of Ischaemia Reperfusion Injury
Post presentation at the Scottish society of Experimental medicine, Edinburgh
November 2008